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Fusaric acid induces mitochondrial stress in human hepatocellular carcinoma (HepG2) cells

By

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DECLARATION

This study represents the original work by the author and has not been submitted in any form to another university. The use of work by others has been duly acknowledged in the text.

The research described in this study was carried out in the Discipline of Medical Biochemistry, Faculty of Health Sciences, University of KwaZulu-Natal, Durban, under the supervision of Professor A.A Chuturgoon and Miss S Nagiah.

A handwritten signature in blue ink, appearing to read 'Naeem Sheik Abdul', is positioned above a horizontal line.

Naeem Sheik Abdul

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My Parents

I would like to thank my parents for raising me, for bearing with me during my mistakes and for joining me in my successes. While most of a child's DNA is a combination from both parents, mitochondrial DNA is inherited maternally, thank you mama for giving me only what a mother can give and for showing me the importance of valuing the people in my life. To my dad, thank you for inspiring me to push beyond my perceived boundaries and for showing me that anything is possible with the right attitude.

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PRESENTATIONS

Fusaric acid induces mitochondrial stress in human hepatocellular carcinoma (HepG2) cells

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TABLE OF CONTENTS

Contents

DECLARATION.....	ii
ACKNOWLEDGEMENTS	iii
PRESENTATIONS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	ix
LIST OF TABLES	xii
LIST OF ABBREVIATIONS.....	xiii
ABSTRACT	xvii
INTRODUCTION	xviii
1.1. Background.....	xviii
1.2. Problem statement	xx
1.3. Aim	xxi
1.4. Research questions	xxi
1.5. Objectives.....	xxi
1.6. Hypothesis.....	xxi
1.7. Experimental approach	xxi
CHAPTER 1: LITERATURE REVIEW	1
1.1. Mycotoxins	1
1.1.1. Characteristics	1
1.1.2. Biological effects of mycotoxins.....	2
1.2. Liver	4
1.2.1. Liver functions and structure	4
1.2.2. Hepatotoxicity	5
1.2.3. Use of the HepG2 cell line	6
1.3. Picolinic acid	6
1.3.2. Mechanism of action.....	7
1.3.3. Pharmacology.....	8
1.4. Fusaric acid.....	9
1.4.1. Toxic effects and mechanism of action	10

1.4.2. Pharmacology.....	12
1.5. Mitochondria.....	13
1.5.2. Mitochondrial biogenesis.....	15
1.5.3. Mitochondrial stress responses	18
1.5.4. Mitochondrial function is regulated by SIRT3	21
1.6. Cellular responses to oxidative stress	25
1.7. Mitochondria in hepatotoxicity.....	27
CHAPTER 2: MATERIALS AND METHODS	29
2.1. Materials	29
2.2. Cell culture and exposure protocol	29
2.2.1. Cell culture	29
2.2.2. Exposure protocol	29
2.3. Metabolic activity.....	30
2.3.1. MTT assay.....	30
2.3.2. ATP assay.....	32
2.4. Oxidative stress – Lipid peroxidation	33
2.4.1. Introduction.....	33
2.4.2. Protocol	34
2.5. Protein expression – western blot	36
2.5.1. Introduction.....	36
2.5.2. Protocol	40
2.6. Gene expression – Quantitative polymerase chain reaction	41
2.6.1. Introduction.....	41
2.6.2. Protocol	43
2.7. Assessment of caspase activity	44
2.7.1. Introduction.....	44
2.7.2 Protocol	44
2.8. Cytotoxicity – lactate dehydrogenase assay (LDH) assay.....	45
2.8.1. Introduction.....	45
2.8.2 Protocol	46
2.9 DNA damage – Single Cell Gel Electrophoresis (SCGE)	46
2.9.1 Introduction.....	46
2.9.2 Protocol	47

2.10. Statistical analyses	48
CHAPTER 3: RESULTS.....	50
3.1. Mitochondrial Output	50
3.1.1. Cell viability	50
3.1.2. Intracellular ATP levels	50
3.2. Oxidative stress and detoxification	51
3.2.1. Oxidative stress	51
3.2.2. Phase 2 detoxification	52
3.3. Mitochondrial stress	53
3.3.1. Sirtuin 3	53
3.3.2. Lon Protease.....	54
3.4. Mitochondrial biogenesis.....	55
3.4.1. Peroxisome proliferator-activated receptor γ co-activator α (PGC-1 α)	55
3.4.2. Phosphorylated cAMP response element binding protein (p-CREB).....	56
3.4.3. Nuclear respiratory factor 1 (NRF-1).....	57
3.4.4. Heat shock protein 70 (HSP70)	58
3.5. Cell damage and death.....	59
3.5.1. Membrane integrity and cytotoxicity.....	59
3.5.2. Caspase activity	60
CHAPTER 4: DISCUSSION	61
CHAPTER 5: CONCLUSIONS	67
5.1. Limitations of study.....	67
5.2. Conclusion	67
5.3. Future studies.....	68
REFERENCES	69
Appendix 1: Raw data for MTT.....	76
Appendix 2:Caspase activity graphs.....	77
Appendix 3: Standard curve for protein isolation.....	78
Appendix 4: SCGE results	79

LIST OF FIGURES

Preface

Figure 1: A schematic showing the experimental approach followed to investigate the effects of FA in cultured HepG2 liver cells (prepared by author).

Chapter 1

Figure 1.1: Biological activities and inhibition sites of mycotoxins (Kiessling 1986)

Figure 1.2: Basic structure of the liver (prepared by author).

Figure 1.3: Mechanisms of hepatotoxicity (prepared by author).

Figure 1.4: In vivo synthesis of PA via the kynurine pathway (Grant et al., 2009)

Figure 1.5: Chemical structures of the isomers Picolinic acid and Nicotinic acid (Grant et al., 2009)

Figure 1.6: Structure of PA, FA and Niacin (Adapted from Ogata, 2001).

Figure 1.7: Ferrous iron in complex with FA (Hirai et al., 2005).

Figure 1.8: The electron transport chain.

Figure 1.9: The number of complex subunits encoded by mitochondrial (mtDNA) and nuclear (nDNA) genomes (Yoboue and Devin, 2012)

Figure 1.10: Structure of SIRT3 in complex with a substrate (Nogueiras et al., 2012)

Figure 1.11: Function and regulation of SIRT3 (prepared by author).

Figure 1.12: General mechanism for the recognition and degradation of protein substrates by LON (Venkatesh et al., 2012).

Figure 1.13: Role of zinc in mediating Nrf2 release (Dinkova-Kostova et al., 2005)

Chapter 2

Figure 2.1: Production of reducing equivalents by the Krebs's cycle

Figure 2.2: Schematic representation of the principle behind the MTT assay (prepared by author).

Figure 2.3: Principle of the CellTire Glo™ assay used to quantify intracellular ATP concentration (prepared by author).

Figure 2.4: Chain reaction of lipid peroxidation. (Ayala et al., 2014).

Figure 2.5: Schematic representation of the TBARS assay principle (prepared by author).

Figure 2.6: Principle of the BCA assay used to quantify protein concentration (prepared by author).

Figure 2.7: Separation of proteins according to size (prepared by author).

Figure 2.8: Transfer of proteins from gel to membrane (prepared by author).

Figure 2.9: Antibody/Antigen signal detection (prepared by author)

Figure 2.10: Steps of the PCR cycle leading up to DNA amplification (prepared by author).

Figure 2.11: Fluorescence when GR dye molecules bind to dsDNA (prepared by author).

Figure 2.12: Schematic of the caspase activity assay (prepared by author).

Figure 2.13: Reactions of the LDH cytotoxicity assay (prepared by author).

Figure 2.14: Principle of the SCGE assay (prepared by author).

Chapter 3

Figure 3.1: A dose dependent decline in metabolic activity following treatment with FA.

Figure 3.2: Intracellular ATP levels were significantly depleted ($p=0.0062$) in FA treated cells.

Figure 3.3: FA induced oxidative stress as seen indicated by elevated MDA levels when compared to NAM and the untreated control ($p = 0.0002$).

Figure 3.4: The regulator of phase II detoxification responses (Nrf2) was found to be significantly elevated in FA treated cells when compared to NAM and the untreated cells ($p < 0.0014$).

Figure 3.5: Protein expression of SIRT3 (A, $p = 0.0012$) and mRNA (B, $p = 0.0007$) were significantly decreased in response to FA stimulation.

Figure 3.6: LON protein expression was significantly down-regulated in cells treated with FA and NAM ($p = 0.0044$).

Figure 3.7: FA significantly decreased the expression of PGC-1 α ($p = 0.0005$).

Figure 3.8: The expression of p-CREB was significantly down-regulated in response to both FA and NAM treatment ($p = 0.0008$).

Figure 3.9: Both the FA and NAM stimulated cells exhibited significantly decreased protein expression of NRF-1 when compared to untreated cells ($p = 0.0004$).

Figure 3.10: The expression of HSP70 was significantly decreased in response to FA stimulation when compared to both NAM and the untreated control cells ($p = 0.0102$).

Figure 3.11: FA caused significant membrane damage and is cytotoxic to the HepG2 cell line as indicated by the LDH assay ($p < 0.0001$).

LIST OF TABLES

Chapter 1

Table 1: Cellular localisation of sirtuins (Parihar et al., 2015).

Chapter 3

Table 2: Caspase activity in FA treated cells. Executioner caspases were significantly up-regulated in FA treated cells (p= 0.0032).

LIST OF ABBREVIATIONS

1-OAADR	1-O-acetyl-ADP-ribose
AceCS2	Acetyl-CoA synthetase 2
acetyl-AMP	Acetyl-adenosine monophosphate
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AMPK	Adenosine monophosphate activated protein kinase
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BHT	Butylated hydroxytoluene
BIR domain	Baculovirus inhibitor of apoptosis protein repeat domain
c DNA	Complementary DNA
CCM	Complete culture medium
CREB	Cyclic AMP (cAMP) response binding element protein
Ct	Cycle time
Cu ⁺	Cuprous ion
Cu ²⁺	Cupric ion
DAS	Diacetoxyscirpenol
DBH	Dopamine-β-hydroxylase
DMSO	Dimethyl sulphoxide
dNTPs	Deoxynucleotide triphosphates
ds	Double stranded
ERRα	Estrogen-related receptor α

ETC	Electron transport chain
FA	Fusaric acid
FADH ₂	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization
Fe ²⁺	Ferrous iron
g	Gravitational force
h	Hours
H ₂ O ₂	Hydrogen peroxide
HBC	Hepatitis C virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HDACS	Histone deacetylases
HIV	Human immunodeficiency virus
HMGCS2	3-hydroxy3-methylglutaryl CoA synthase 2
HNSCC	Head and neck squamous cell cancer
HRP	Horseradish peroxidase
IAP	Inhibitors of apoptosis proteins
IC ₅₀	Median inhibition concentration
ICDH	Isocitrate dehydrogenase
IFN γ	Interferon γ
Keap1	Kelch-like ECH-associated protein 1
KYNA	Kynurenic acid
LCAD	Long-chain acyl-CoA dehydrogenase
LDH	Lactate dehydrogenase assay
LMPA	Low melting point agarose
LON	Lon protease

LPO	Lipid peroxidation
MDA	Melandaldehyde
MIP	Macrophage inflammatory proteins
MPT	Mitochondrial permeability transition
mRNA	messenger RNA
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
nDNA	Nuclear DNA
NFDM	Non-fat dry milk
NMN	Nicotinamide mononucleotide
NOS	Nitric oxide synthase
NRF1/NRF2	Nuclear respiratory factor 1/2
Nrf2	Nuclear factor-erythroid 2-related factor 2
OH ⁻	Hydroxyl radical
OXPHOS	Oxidative phosphorylation
PA	Picolinic acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGC-1	Peroxisome proliferator-activated receptor gamma co-activator
RBD	Relative band density
RLU	Relative light units
RNA	Ribose nucleic acid
ROS	Reactive oxygen species

RT	Room temperature
SCGE	Single Cell Gel Electrophoresis
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SIRT	Sirtuins
Smac/DIABLO	Second mitochondria-derived activator caspases/DIABLO
SOD	Superoxide dismutase
ss	Single stranded
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
Tfam	Mitochondrial transcription factor A
TTBS	Tris-buffer saline
XIAP	X-linked mammalian inhibitor of apoptosis protein

ABSTRACT

Fusarium spp are common contaminants of maize and produce many mycotoxins, including the fusariotoxin fusaric acid (FA). FA is a niacin related compound, chelator of divalent cations, and mediates toxicity via oxidative stress and possible mitochondrial dysregulation. Sirtuin 3 (SIRT3) is a stress response deacetylase that maintains proper mitochondrial function. The effect of FA on SIRT3 and oxidative and mitochondrial stress pathways in the hepatocellular carcinoma (HepG2) cell line were investigated. We determined FA toxicity (24h incubation; IC_{50} = 104 μ g/ml) on mitochondrial output, cellular and mitochondrial stress responses, mitochondrial biogenesis and markers of cell death using spectrophotometry, luminometry, qPCR and western blots. FA caused a dose dependent decrease in metabolic activity along with significant depletion of intracellular ATP ($p = 0.0062$). FA induced a significant increase in lipid peroxidation ($p = 0.0002$), despite up-regulation of the anti-oxidant transcription factor, Nrf2 ($p < 0.0014$). FA significantly decreased expression of SIRT3 mRNA ($p = 0.0007$) with a concomitant decrease in protein expression ($p = 0.0012$). Lon protease was also significantly down-regulated ($p = 0.0044$). FA induced aberrant mitochondrial biogenesis as evidenced by significantly decreased protein expressions of: PGC-1 α ($p = 0.0005$), p-CREB ($p = 0.0008$), NRF1 ($p = 0.0004$) and HSP70 ($p = 0.0102$). Finally, FA activated apoptosis as noted by the significantly increased activity of caspases 3/7 ($p = 0.0032$) and also induced cellular necrosis ($p < 0.0001$). This study provides insight into the molecular mechanisms of FA (a neglected mycotoxin) induced hepatotoxicity and may aid in understanding and predicting the contamination risks of FA. This is of particular importance to developing countries where the risk of mycotoxin exposure is high due to consumption of often contaminated agricultural produce.

INTRODUCTION

1.1. Background

Third world countries, particularly those found in Africa are faced with a multitude of socio-economic struggles making it one of the most food insecure regions. The Food and Agriculture Organization (FAO) defines food security as “a situation that exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food to meet their dietary needs and food preferences for an active and healthy life” (Schmidhuber and Tubiello, 2007). But in Africa, the challenge to meeting the above mentioned criteria are hampered by an ever increasing population and intense poverty often leading to the consumption of contaminated food.

Maize is an important food source for millions of South Africans (Boutigny et al., 2011). Fungi under suitable conditions parasitize agricultural produce including maize. These fungi produce secondary metabolites known as mycotoxins that benefit the fungi by being toxic to other organisms thereby acting as a survival mechanism (Hussein and Brasel, 2001). Maize is infected by a number of fungal pathogens including members of the genera *Fusarium*. The growing interest in *Fusarium* species stems from their ability to produce fusariotoxins (Nedělník, 2002).

Acute and chronic effects are implicated in diseased states (Coulombe, 1993) and is dependent on the species and susceptibility of the animal to the toxin (Pal et al., 2015). Acute toxicity results in a rapid onset of toxin induced responses while chronic exposure is characterized by low levels of toxin over a long time period often resulting in irreversible damage and carcinogenesis (Pal et al., 2015). An often neglected fusariotoxin is fusaric acid (FA), a niacin (i.e. nicotinamide) related compound (Ogata et al., 2001) and efficient chelator of divalent cations (Hirai et al., 2005, Stack et al., 2004).

Fusaric acid (5-butylpicolinic acid) is a derivative of picolinic acid and is produced mainly by *Fusarium moniliforme* (Nedělník, 2002), it possesses only low to moderate toxicity and has several pharmacological properties evident in the cardiovascular, immune and nervous systems (Stack et al., 2004, Wang and Ng, 1999). FA enhances the toxicity of other mycotoxins and this synergism is considered the major contributor to its mechanism of action (Malovrh and Jakovac-Strajn, 2010, Fairchild et al., 2005). These synergistic effects can be attributed to the presence of a plethora of *Fusarium* spp. and strains within the contaminated feed samples or due to multiple mycotoxins being produced by the same species (Bacon et al., 1996). Regardless, an analysis focusing on a single toxin is likely to show better correlation between its concentration

and induced toxicity. Mycotoxins exert a variety of biological effects owing to their diverse chemical structures, thus no generalized mechanism of action can be applied to all mycotoxins (Kiessling, 1986).

Several studies on FA showed it to induce oxidative stress by down regulating anti-oxidant enzymes and increasing the production of reactive oxygen species (ROS) (Iwahashi et al., 1999, Jiao et al., 2014, Singh and Upadhyay, 2014). Excess ROS induces oxidative modifications to macromolecules, inhibits protein functions, promotes cell death (Circu and Aw, 2010) and has been implicated in disease initiation and progression (Qiu et al., 2010). Although ROS can be generated in many cellular compartments, the mitochondria is the major contributor (Balaban et al., 2005). ROS formation occurs when unpaired electrons escape the electron transport chain (ETC) and react with molecular oxygen (St-Pierre et al., 2006). FA may exert its toxic effects through dysregulation of mitochondrial processes (Telles-Pupulin et al., 1998) leading to excess ROS generation.

Sirtuin 3 (SIRT3) is an important regulator of mitochondrial lysine acetylation (Lombard et al., 2007). SIRT3 deacetylase activity is stimulated by its co-factor, nicotinamide adenine dinucleotide (NAD^+) and inhibited by its reaction product, nicotinamide (NAM) (Guan et al., 2014). SIRT3 influences homeostasis by targeting enzymes that regulate key mitochondrial processes including the Krebs' cycle (Finley et al., 2011) and oxidative phosphorylation (OXPHOS) (Ahn et al., 2008). SIRT3 also maintains mitochondrial integrity by regulating the stress response protein LON protease (LON) at the post-translational level (Gibellini et al., 2014a). LON is an ATP dependent protease that catalyzes the degradation of oxidatively damaged proteins in the mitochondrial matrix. LON can also act as a chaperone protein, independent of its proteolytic activity and promotes the assembly of ETC subunits (Bota et al., 2005).

The primary defense against oxidative stress are endogenous anti-oxidant enzymes that scavenge excess ROS. The transcriptional co-activator, peroxisome proliferator-activated receptor gamma co-activator α (PGC-1 α) interacts with various transcription factors to regulate biological programs (Lin et al., 2005). PGC-1 α regulates ROS metabolism by mediating the expression of anti-oxidant enzymes catalase and superoxide dismutase (SOD) (St-Pierre et al., 2006). Oxidative stress increases the expression of both PGC-1 α , and SIRT3 expression through activation of estrogen-related receptor α (ERR- α) In turn, SIRT3 stimulates PGC-1 α expression *via* phosphorylated cAMP response element binding protein (pCREB), thereby forming a positive feedback loop and increasing anti-oxidant defences (Kong et al., 2010). SIRT3 is also

known to enhance SOD2 activity (the primary mitochondrial superoxide detoxification enzyme) by deacetylation (Tao et al., 2010, Qiu et al., 2010).

The induction of the transcription factor nuclear factor-erythroid 2-related factor (Nrf2) and subsequent mediation of phase 2 response is an important cellular reaction to oxidative stress and is dependent on the release of Nrf2 from its repressor Kelch-like ECH-associated protein 1 (Keap1), a zinc thiol protein (Dinkova-Kostova et al., 2005). It has been suggested that Nrf2 is a transcriptional regulator of SIRT3 (Flick and Lüscher, 2012) and LON (Ngo et al., 2013).

Mitochondrial biogenesis is a mechanism developed by cells to prevent mitochondrial lesions and maintain mitochondrial integrity (Esposti et al., 2012). PGC-1 α is the master regulator of mitochondrial biogenesis and respiration (St-Pierre et al., 2006) by mediating the activity of several transcription factors including the transcription factor nuclear respiratory factor 1 (NRF-1) (Yoboue and Devin, 2012). Furthermore, SIRT3 was shown to mediate the effects of PGC-1 α on mitochondrial biogenesis (Kong et al., 2010).

The liver is constantly being challenged by various stimuli making it a frequent site for damage from toxic insults. Toxins reach the liver in concentrated forms since ingested materials are first processed by the liver before entering systemic circulation. Furthermore, most detoxification reactions are carried out mainly by the liver ameliorating toxicity (Fenton, 2002). Furthermore, the high density of mitochondria within liver cells is key to cell survival, with even mild mitochondrial dysfunction resulting in oxidative stress and cell death. The liver derived HepG2 cell line is widely used as an *in vitro* toxicity model that reflects xenobiotic metabolism owing to inducible expression of detoxification enzymes (Mersch-Sundermann et al., 2004).

Although a common contaminant of agricultural produce little is known about the underlying molecular mechanisms of FA induced oxidative stress and mitochondrial dysfunction. We investigated the effects of the niacin related compound, FA, on SIRT3 and its ability to induce oxidative and mitochondrial stress in the HepG2 liver cell line.

1.2. Problem statement

Maize is the staple diet of majority population in South Africa and is also used as feed for livestock. This puts the public at risk of exposure to mycotoxins in particular those produced by the *Fusarium spp.* (ubiquitous soil fungi). Many studies have been carried out on various fusariotoxins, but to date, little work has been done on FA. Although chelation and induction of oxidative stress have been cited as general mechanisms of FA toxicity, molecular pathways mediating its mechanisms of action are yet to be elucidated.

1.3. Aim

The aim of this study was to investigate the mechanism of FA cytotoxicity and mitochondrial toxicity in the HepG2 liver cell line.

1.4. Research questions

This study focused specifically on the cytotoxic and mitochondrial toxic effects of FA in a liver cell line. The liver is the main site of xenobiotic metabolism and is densely populated with mitochondria making it prone to toxic insult by FA. The following research questions were posed:

- Does FA interfere with liver cell metabolism and mitochondrial output?
- Does FA induce liver cell mitochondrial stress?
- Does FA affect liver cell mitochondrial biogenesis?

1.5. Objectives

- Determine metabolic activity of liver cells treated with FA
- Determine molecular mechanisms involved in the dysregulation of mitochondrial stress responses in liver cells treated with FA

1.6. Hypothesis

It was hypothesised that FA (a weak acid) induced mitochondrial toxicity in HepG2 cells by mediating dysregulation of key proteins involved in mitochondrial function and stress responses.

1.7. Experimental approach

The human HepG2 hepatocellular carcinoma cell line was used as a model to investigate the mitochondrial toxic properties of FA. Cells were treated for 24 hours (h) with a range of FA concentrations to determine cell viability and the concentration that yielded a 50% reduction in viability (IC_{50} ; deduced from the dose-response curve). All assays to determine mitochondrial toxicity and cell damage used this IC_{50} value (Figure 1).

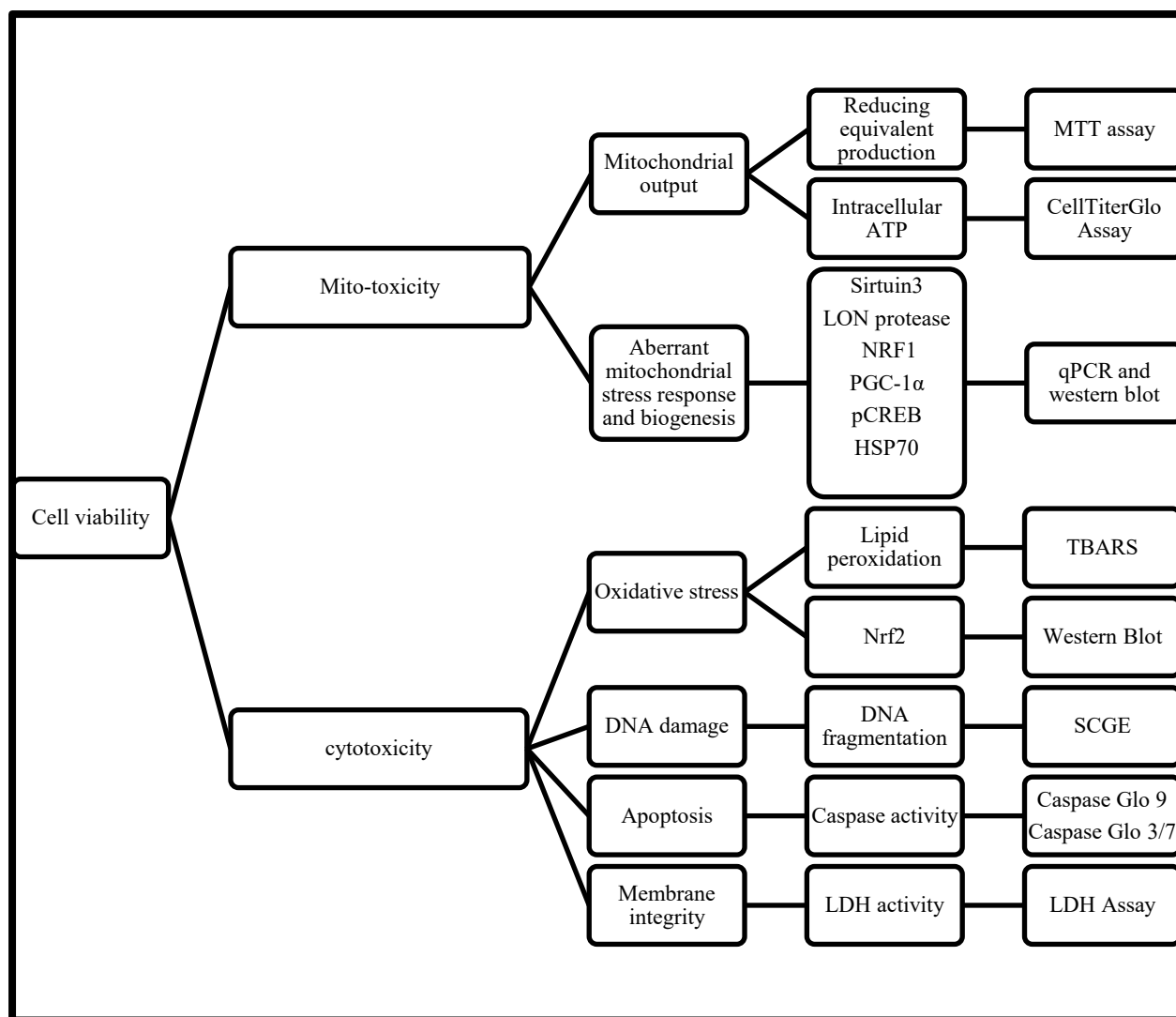


Figure 1: A schematic showing the experimental approach followed to investigate the effects of FA in cultured HepG2 liver cells; Adenosine triphosphate (ATP), Heat shock protein 70 (HSP70), Lactate dehydrogenase (LDH), Metyhyl thiazol tetrazolium (MTT), Nuclear factor-erythroid 2-related factor 2 (Nrf2), Nuclear respiratory factor 1(NRF-1), Peroxisome proliferator-activated receptor gamma co-activator 1 α (PGC-1), phosphorylated Cyclic AMP response binding element protein (pCREB), Quantitative polymerase chain reaction (qPCR), Single cell gel electrophoresis (SCGE), Thiobarbituric acid reactive substances (TBARS) (prepared by author).

CHAPTER 1: LITERATURE REVIEW

1.1. Mycotoxins

1.1.1. Characteristics

Mycotoxins are mould secondary metabolites produced when these fungi contaminate food and feed. One species of mould can produce a plethora of mycotoxins and the same mycotoxin can be produced by a variety of mould species. The severity of contamination is dependent on environmental factors as well as storage methods with humid conditions accelerating the rate of contamination (Coulombe, 1993). Ingestion of mycotoxins at various steps of the food chain (Zain, 2011) leads to an array of toxicity in both animals and humans.

Acute and chronic effects are implicated in diseased states (Coulombe, 1993) and is dependent on the species and susceptibility of the animal to the toxin (Pal et al., 2015). Acute toxicity results in a rapid onset of toxin induced responses while chronic exposure is characterized by low levels of toxin over a long period of time often resulting in irreversible damage and carcinogenesis. Exposure to mycotoxins is mainly through ingestion of contaminated agricultural produce or consuming animal products containing mycotoxin metabolites, however, inhalation of fungal spores and dermal contact have also been cited as possible exposure routes (Zain, 2011, Pal et al., 2015).

Secondary metabolites are structurally diverse, low molecular weight compounds with obscure functions, and unlike primary metabolites, which are universally distributed compounds of intermediary metabolism (Zain et al., 2013), secondary metabolites are not directly required to facilitate growth of the producing organism (Fox and Howlett, 2008). Regulation of secondary metabolism in fungi is complex, responding to environmental cues and host stimuli (Fox and Howlett, 2008). Most secondary metabolites are synthesized from a few precursors which branch from a limited number of primary metabolism reaction points (Zain et al., 2013). Secondary metabolites play roles in reproduction and differentiation and are often associated with providing the producing organism with a competitive advantage allowing them to survive in their ecological niche (Fox and Howlett, 2008) through inhibiting the growth of other organisms in the environment (Zain et al., 2013).

1.1.2. Biological effects of mycotoxins

While all mycotoxins are fungal in origin, not all toxic compounds produced are termed mycotoxins. The cellular target and concentration of the metabolite are both important (Zain, 2011). Mycotoxicoses presents with symptoms resulting from the interactions of functional molecules and organelles with mycotoxins. Mycotoxins exert a variety of biological effects owing to their diverse chemical structures, thus no generalized mechanism of action can be applied to all mycotoxins (Kiesling, 1986).

Mycotoxins exert their biological effects by altering basic cellular processes including mitochondrial function, carbohydrate metabolism, lipid and steroid metabolism as well as the biosynthesis of protein and DNA (Figure 1.1). A mycotoxin may act primarily on the DNA template, or impair and inhibit transcription and translation processes thus interfering with protein synthesis. These primary events may then result in secondary effects in which metabolic processes are altered due to aberrant enzyme activity and regulation (Kiesling, 1986).

Many mycotoxins act at the level of DNA and RNA processing with several showing carcinogenic potential. Strong correlations can be established between carcinogenicity and the extent of covalent DNA binding among mycotoxins such as aflatoxin B₁ and its related compounds. These covalent bonds often cause mutations leading to cancer. Mycotoxins are able to inhibit DNA synthesis and induce both single and double strand breaks. The inhibition of RNA polymerase and decrease in RNA content by mycotoxins (Kiesling, 1986) show that these metabolites are able to affect transcriptional processes.

Inhibitory effects of mycotoxins on transcriptional and translational processes, including those of the mitochondria (Bin-Umer et al., 2011) may result in the inhibition of protein synthesis and enzyme activity resulting in modified cellular processes and metabolism. A vast array of mycotoxins has been shown to adversely affect mitochondria. This is achieved through the inhibition of the Krebs's cycle, uncoupling of oxidative phosphorylation and through competitive inhibition of mitochondrial transport proteins (Kiesling, 1986). Furthermore mycotoxins were shown to increase reactive oxygen species (ROS) generation and decrease mitochondrial membrane potential (Bin-Umer et al., 2011).

Numerous mycotoxins are implicated in the dysregulation of lipid metabolism, aflatoxin B₁ is able to affect transport of triglycerides but also of phospholipids and cholesterol (Kiesling, 1986).

At the organ level, mycotoxins have been shown to stimulate immune activity but inhibit immune cell proliferation resulting in an incompetent immune response. Mycotoxins are also known to cause constriction of the small airways. But the most threatening effect of mycotoxin exposure is the risk of carcinogenesis and mutagenesis, particularly in metabolizing organs such as the liver and kidney (Pal et al., 2015).

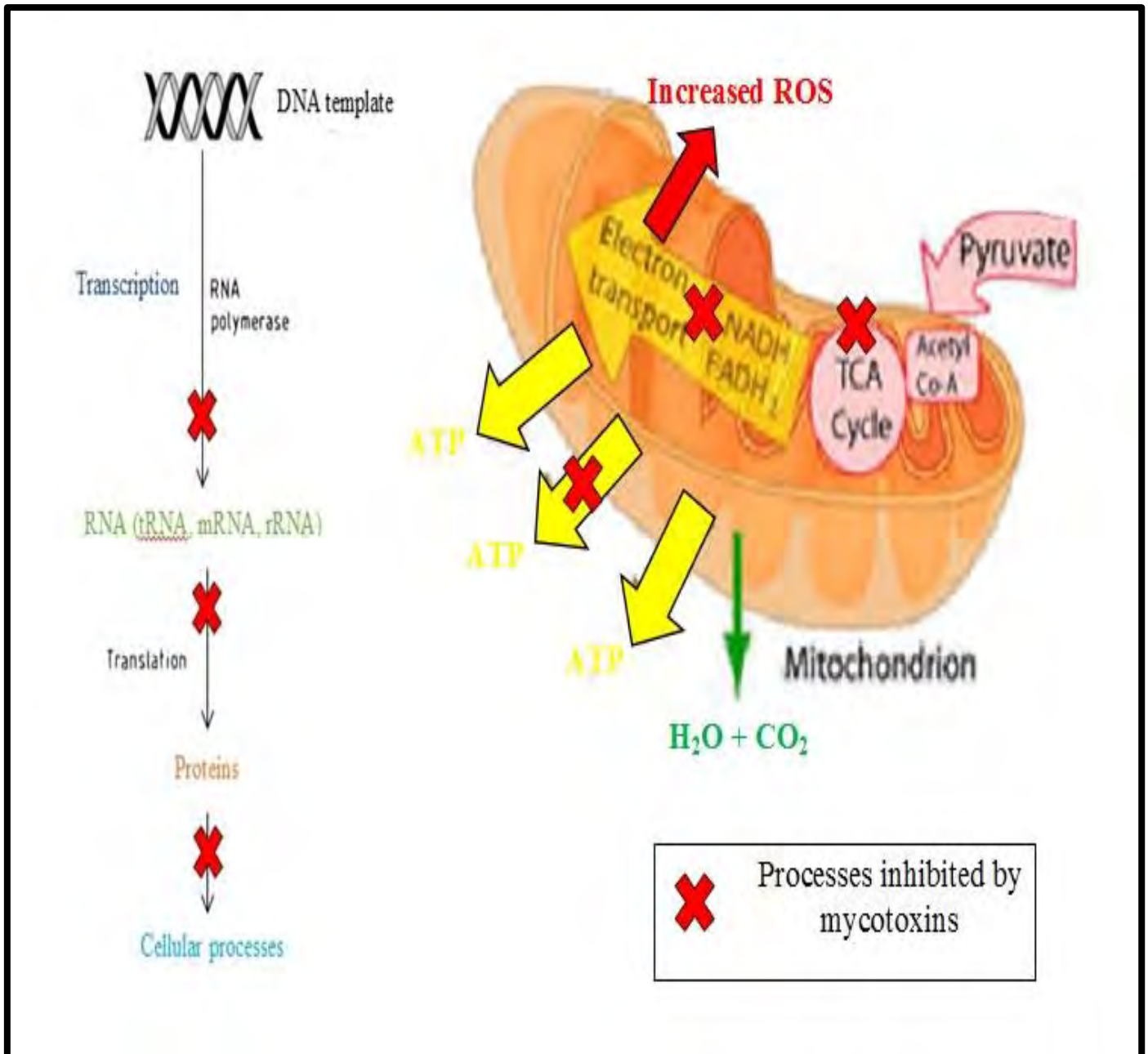


Figure 1.1: Mycotoxins exert a variety of biological activities and inhibit various cellular processes (adapted from Kiessling, 1986).

1.2. Liver

1.2.1. Liver functions and structure

The liver serves a multitude of functions and plays key roles in metabolism, secretion, excretion and immune responses. The major metabolic and synthetic functions of the liver include carbohydrate, lipid and protein metabolism. Synthesis of proteins, mainly albumin takes place in the liver as well as the excretion of exogenous compounds such as xenobiotics and endogenous substances like bilirubin. The liver also secretes bile which is needed for the absorption of fat and fat soluble vitamins in the small intestine (Mitra and Metcalf, 2009). The immunological functions of the liver are executed by hepatic macrophages known as Kupffer cells (Fenton, 2002).

The liver is composed of highly metabolic cells. Liver cells are called hepatocytes and are organized in rows forming lobules (Campbell, 2006) adjacent to sinusoids. Sinusoids are spaces that are supplied by branches of the hepatic artery and portal vein which in turn supply hepatocytes with solutes from blood (Figure 1.2). These solutes bathe the hepatocytes which absorb many of the dissolved particles (Fenton, 2002)

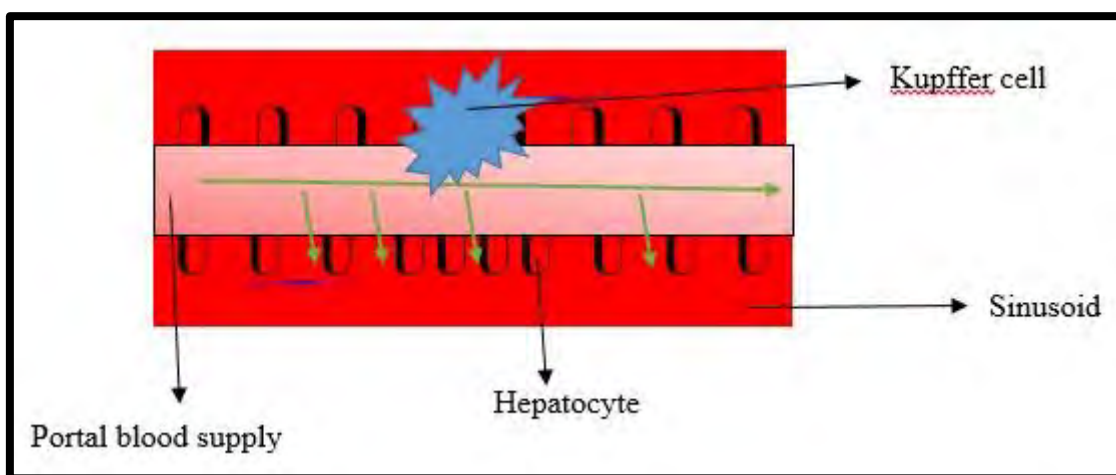


Figure 1.2: Basic structure of the liver (prepared by author).

Hepatocytes are rich in smooth and rough endoplasmic reticulum which is necessary to carry out xenobiotic metabolism and protein synthesis respectively (Campbell, 2006). The liver also possesses a high number and density of mitochondria which form an integrative hub of carbohydrate, lipid and protein metabolism as well as being essential regulators of hepatocyte death and survival. The large number of mitochondria is essential for energy and ROS

production the latter being needed for cellular signaling, fine tuning responses to stress and global adaptations to metabolism (Esposti et al., 2012).

The liver is the primary site of xenobiotic metabolism. Most compounds are not intrinsically toxic to the liver and require metabolic transformation into a toxic metabolite via a process known as bioactivation. Since gastrointestinal absorption is enhanced by lipophilicity of a compound the liver is essential to the conversion of these compounds to more water soluble metabolites that can be excreted (Sturgill and Lambert, 1997). There are two types of reactions involved in xenobiotic metabolism: phase I and phase II reactions. Phase I reactions occur first and involve oxidation, hydrolysis or reduction of the xenobiotic and enhance water solubility by generating hydroxyl, carboxyl, or epoxide functional groups on the parent compound. These functional groups can then facilitate phase II reactions which further enhance water solubility through conjugation with glucuronate, sulphate or other glutathione moieties (Sturgill and Lambert, 1997).

1.2.2. Hepatotoxicity

The severity of xenobiotic toxicity is largely determined by the organisms' rate of metabolism (Swick, 1984). Toxic chemicals frequently damage the liver as the portal vein carries ingested material directly to the liver in a relatively concentrated form. Furthermore toxins are concentrated in the liver since most detoxification reactions occur in this organ, included amongst these reactions is bioactivation in which the pre-toxin is converted to a toxic form by metabolic enzymes (Fenton, 2002). Injury to hepatocytes can be cytotoxic (morphological changes to cells), genotoxic (damage to DNA) or metabolic (affecting cell metabolism and mitochondria) (Figure 1.3) (Castell et al., 1997); and is dependent on the toxin as well as the degree and frequency of exposure. All of these factors contribute to hepatotoxicity and culminate in aberrant cell survival processes leading to necrotic hepatocyte death or carcinogenesis (Fenton, 2002).

Hepatocellular carcinoma (HCC) is the primary malignancy of the liver (Zhao et al., 2011) and is the fifth most common cancer worldwide. It is a growing malignancy with poor patient survival despite the development of new treatments over the past few decades (Bodzin and Busuttil, 2015).

The epidemiology of HCC is complex and involves a host of factors and co-factors. Chronic infection with the hepatitis B virus and hepatitis C virus (HBV and HCV respectively) are responsible for the majority of cases of HCC cases worldwide. Of concern, particularly in Africa is the consumption of agricultural products infected with mould strains that produce the

aflatoxin. The synergism between HBV and aflatoxin exposure increases the risk of developing HCC. Human immunodeficiency virus (HIV) infection is also thought to increase rates of HCC in HBV-infected individuals and this may be of particular relevance in sub-Saharan Africa, where 67% of all individuals living with HIV reside (Venook et al., 2010, Kew, 2010).

1.2.3. Use of the HepG2 cell line

The liver derived HepG2 cell line is widely used as an *in vitro* toxicity model that reflects xenobiotic metabolism owing to inducible expression of phase I and phase II enzymes as well as the expression of anti-oxidant systems (Mersch-Sundermann et al., 2004). This suggests that the HepG2 cell line can be used as a suitable cell model to determine stress responses.

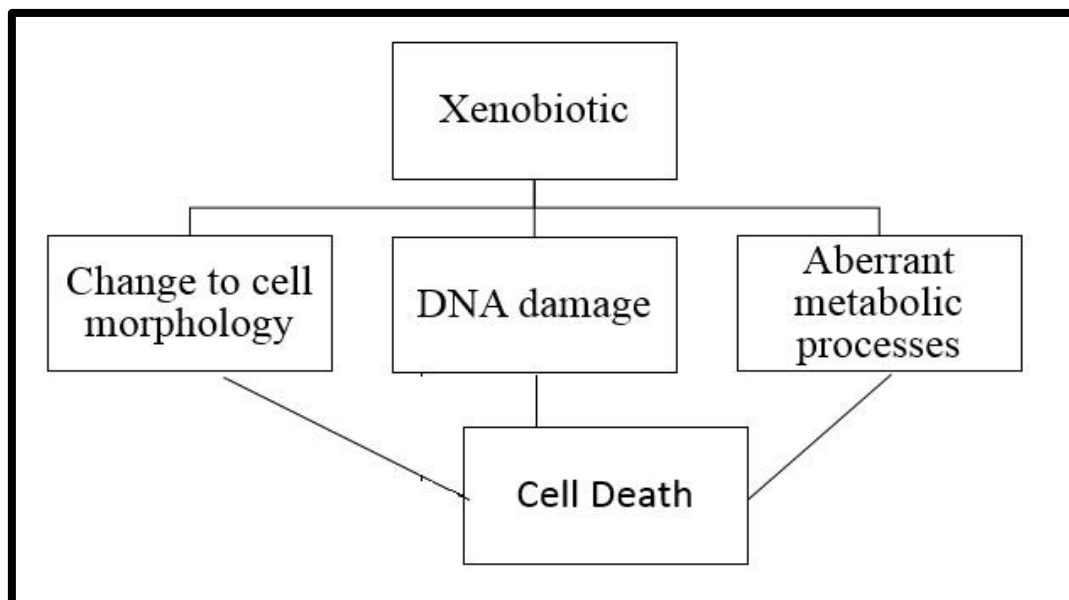


Figure 1.3: Mechanisms of hepatotoxicity (prepared by author).

1.3. Picolinic acid

Picolinic acid (PA) is a six-membered ring structure compound (pyridine ring), containing five carbon atoms, a nitrogen and a carboxyl group at position two (Grant et al., 2009) and is a niacin related compound (Ogata et al., 2000). Picolinic acid is primarily formed in the liver, kidney and brain (Grant et al., 2009) and is synthesized in a side pathway of NAD biosynthesis in animals (Ogata et al., 2001). Tryptophan can be metabolized via the kynurenine pathway which oxidatively degrades this amino acid to yield kynurenic acid (KYNA), PA and NAD (Figure 1.4) (Grant et al., 2009). Picolinic acid is also produced as a toxin by the mould species *Magnorpathegrisea* and *Fusarium spp* contributing to their pathogenesis (Zhang et al., 2004).

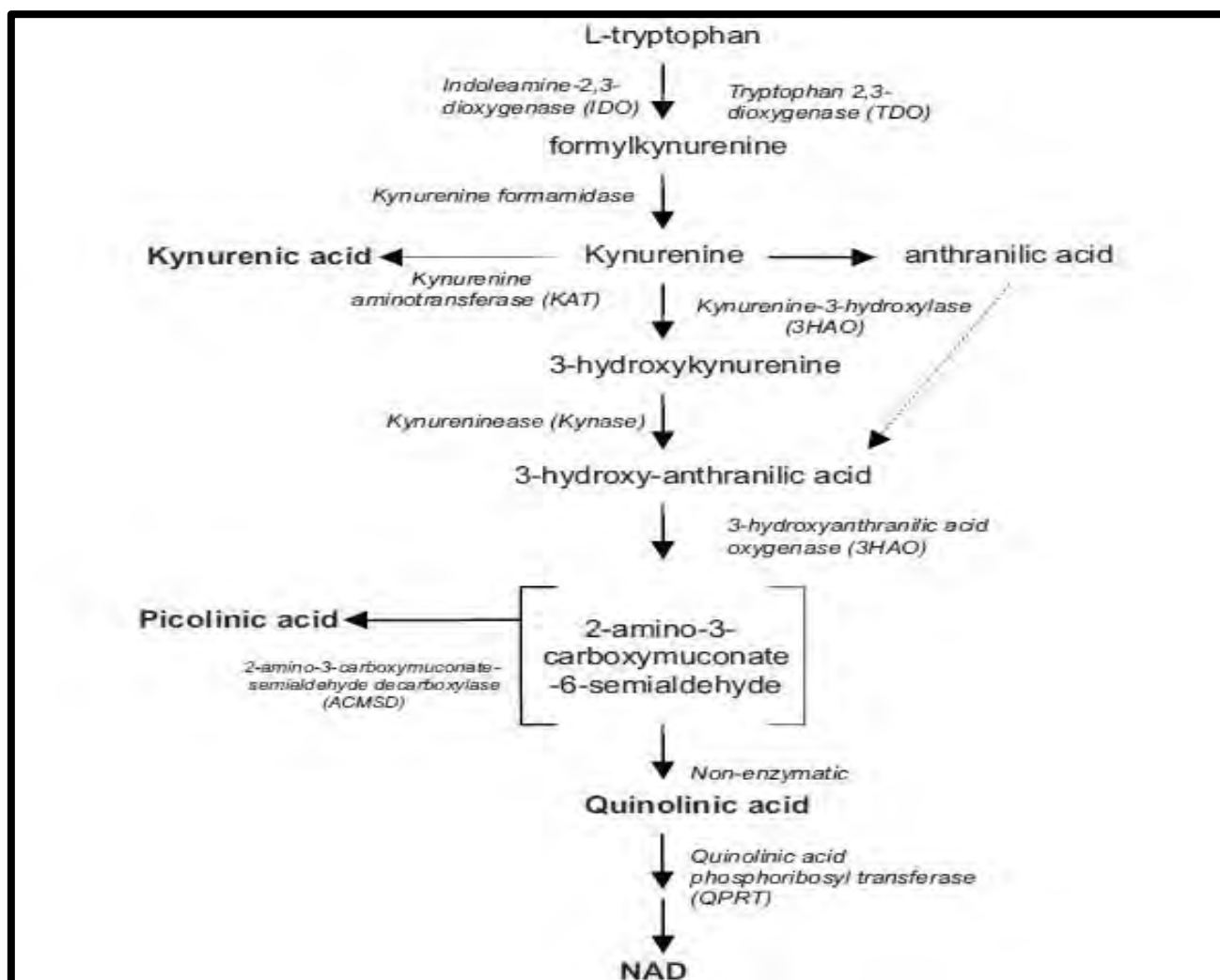


Figure 1.4: In vivo synthesis of PA via the kynurine pathway (Grant et al., 2009)

1.3.2. Mechanism of action

The best characterised physical property of PA is its ability to efficiently chelate divalent metal cations including iron, zinc, copper, nickel and lead (Fernandez-Pol et al., 1977). Picolinic acid is related to niacin (i.e. nicotinic acid, nicotinamide) (Figure 1.5) which is a water soluble vitamin (Ogata et al., 2001) and plays various physiological roles in organisms (Ogata et al., 2000). It can be converted to NAD *in vivo*. NAD is an important co-factor required for DNA repair, energy production (Ogata et al., 2001) and apoptosis (Ogata et al., 2000).

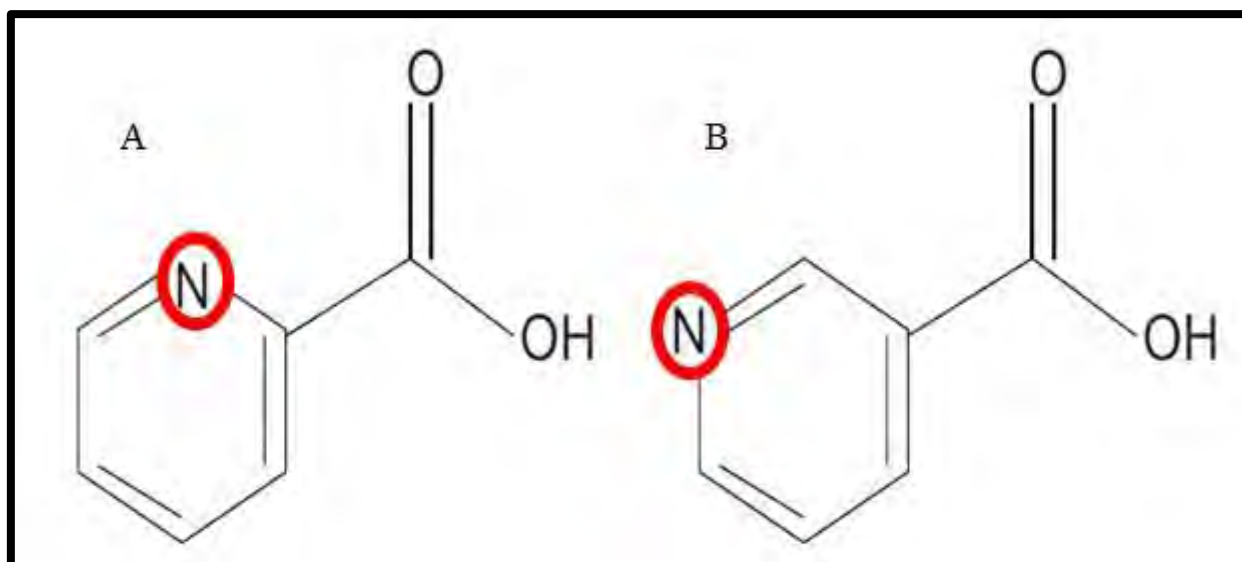


Figure 1.5: Chemical structures of the isomers Picolinic acid (A) and Nicotinic acid (B)
(Grant et al., 2009).

1.3.3. Pharmacology

Exploiting its chelatory capabilities, PA-metal complexes are now used as a means to introduce bioactive metals into biological systems. Chromium-picolinate supplementation has been advocated in type II diabetes. Chromium has effects on lipid and carbohydrate metabolism but is poorly assimilated in the body. The PA in complex with chromium allows for better absorption of this metal (Grant et al., 2009).

In experimental systems PA has been shown to elicit a number of effects within the body, particularly in immune system functioning. Macrophage effector functions were enhanced by PA through the increased gene expression of nitric oxide synthase (NOS) and expression of the macrophage inflammatory proteins (MIP) 1 α and 1 β mediated by interferon γ (IFN γ). Other studies have shown PA to possess anti-viral and anti-bacterial potential (Grant et al., 2009).

Investigators have also observed the effects of PA on cell cycle progression. Fernandez-Pol et al. (1977) showed that PA reversibly inhibits growth of cultured cells. Normal rat kidney cells were arrested in the G1 phase whereas transformed cells were arrested at various stages depending on the virus used to transform them. These results suggest that PA induces cell cycle arrest by interacting with a specific growth control mechanism that may involve NAD⁺ (Fernandez-Pol et al., 1977). Studies have also shown that PA possesses tumouricidal activity. Ogata et al. (1998) demonstrated that PA induces apoptosis and DNA fragmentation in the human promyelocytic leukaemia (HL-60) cell line (Ogata et al., 1998). In human myelogenous leukaemia cells (K562) PA was shown to cause DNA fragmentation and elevate levels of intracellular peroxide; however apoptosis was not observed in normal human quiescent

lymphocytes. This suggests that PA exerts some degree of tumour specificity (Ogata et al., 2000). Most niacin related compounds exist as natural components in organisms, possessing unknown functions and warrant investigation as potential therapeutics (Ogata et al., 2000).

1.4. Fusaric acid

Fusaric acid (5-butylpicolinic acid) is a PA derivative and niacin related compound (Ogata et al., 2001) belonging to a novel class of nicotinic acid derivatives (Stack et al., 2014). The structure of FA is similar to that of PA as both contain pyridine rings, however FA has a substituted butyl group at position 5 (Figure 1.6) (Ogata et al., 2001). Fusaric acid is a common contaminant of maize and maize based food and feeds and is also found in grain where *Fusarium* spp. are isolated. Peculiarly, Bacon et al. (1996) showed that FA is synthesized by all strains of *Fusarium* spp. surveyed. Thus, this secondary metabolite is different from other mycotoxins synthesized by various *Fusarium* spp., e.g. deoxynivalenol, and zearalenone, which are restricted to a few taxonomic groups among a species population. These investigators estimated that 11,665 species of plants, hundreds of which are agriculturally significant food plants, may serve as hosts of *Fusarium* spp. that are capable of producing FA. They concluded that FA is likely to be one of the most widely distributed mycotoxins produced by strains in the genus *Fusarium* (Bacon et al., 1996).

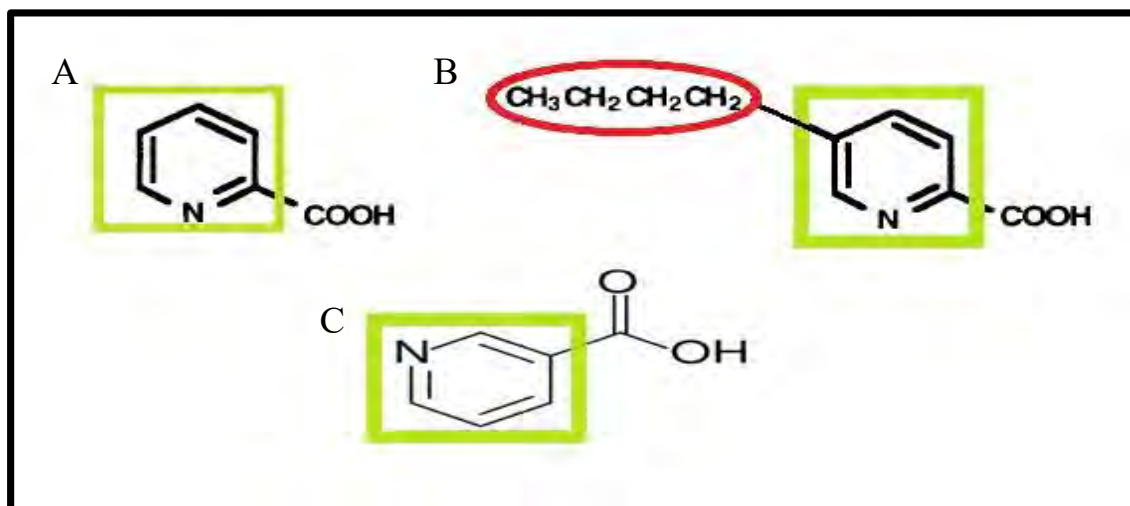


Figure 1.6: Structure of PA (A), FA (B) and Niacin (C). All three molecules are pyridine ring structures (Green box) however FA has a butyl group at position 5 (Red oval). Adapted from (Ogata et al., 2001).

1.4.1. Toxic effects and mechanism of action

Fusaric acid has been classed as a phytotoxin (Smith and MacDonald, 1991) and is known to possess low to moderate toxicity in human and animal models. This is of concern since FA shows synergism with other fusariotoxins (Wang and Ng, 1999). The enhancement of toxicity may be explained by the variety of *Fusarium* spp. and strains within the contaminated feed samples or due to multiple mycotoxins being produced by the same species. Regardless, analyses focusing solely on a single toxin is unlikely to show a good correlation between the concentration of any single mycotoxin and its induced toxicity (Bacon et al., 1996).

1.4.1.1. In vitro toxicity

The effect of FA on the transfer of electrons from iron (ferrous state) to molecular oxygen was investigated by measuring oxygen consumption rates. This study showed that FA is capable of repressing oxygen consumption. This repression is related to the efficiency of the electron transfer from Fe^{2+} -ligand complex to the oxygen molecule (Hirai et al., 2005) suggesting that FA is capable of affecting the ETC.

Experiments by Iwahashi et al (1999) were conducted to clarify the effects of FA on hydroxyl radical (OH^\cdot) formation, and focussed on the interactions between this mycotoxin and iron ions. These investigators found that FA, via Fenton chemistry, enhanced the formation of the hydroxyl radical through chelation of iron ion. It is proposed that the oxygen atom in the carboxyl group and the nitrogen atom in the pyridine ring are responsible for the chelation of Fe^{2+} (Figure 1.7) (Iwahashi et al., 1999).

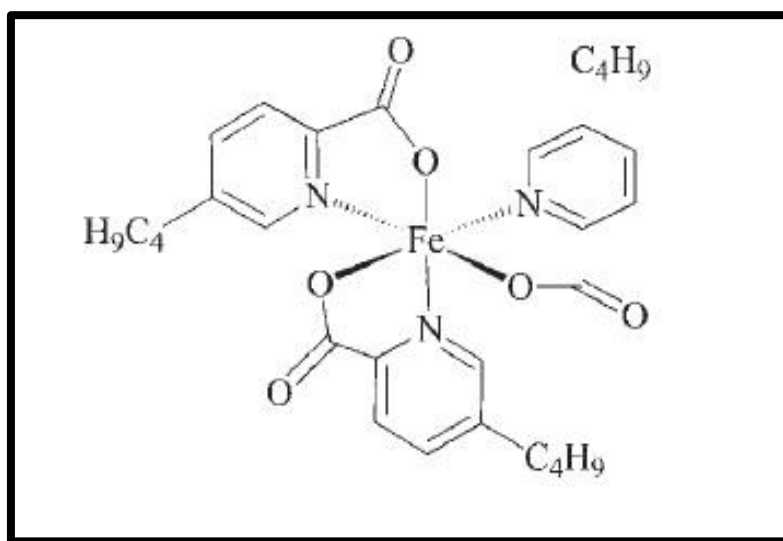


Figure 1.7: Ferrous iron in complex with FA (Hirai et al., 2005).

The toxic effects of FA on rat liver mitochondria were studied by Telles-Pupulin and colleagues (1998). Their study showed that FA affected mitochondrial energy metabolism in at least three ways: (1) Inhibition of succinate-dehydrogenase, (2) inhibition of oxidative phosphorylation, and (3) inhibition of α -ketoglutarate-dehydrogenase. The inhibition of oxidative phosphorylation may be attributed to direct action of FA on the ATP-synthase complex without significantly inhibiting the ATP:ADP exchange (Telles-Pupulin et al., 1998).

1.4.1.2. Animal toxicity

Toxicity studies conducted in primiparous sows during the perinatal period showed that FA in synergism with other mycotoxins (deoxynivalenol and zearalenone) altered lymphocyte proliferation and apoptosis indicating that FA impairs the immune response. Feed intake was also decreased in the treated group (Malovrh and Jakovac-Strajn, 2010). The individual and combined effects of dietary diacetoxyscirpenol (DAS) and FA on turkey performance was determined by Fairchild et al. (2005). Their results showed that individually FA and DAS exhibit less toxicity than when administered together. This study also alluded to a protective effect of FA in DAS induced mouth lesions (Fairchild et al., 2005).

An earlier study by Smith and MacDonald (1991) showed that pigs dosed with FA were lethargic and displayed brain neurochemical changes. This group also observed vomiting in swine treated with FA and concluded that FA induced toxic responses similar to Trichothecenes, proposing synergism of these mycotoxins (Smith and MacDonald, 1991). In a recent study, Yin et al. (2015) described a mechanism of notochord malformation in zebrafish after treatment with FA; an undulated notochord was attributed to chelation of copper by FA leading to aberrant lysyl oxidase activity. This enzyme is dependent on copper as a co-factor and cross-links collagen and elastin by catalysing allysine formation (Yin et al., 2015). These results implicate FA as a possible teratogen.

1.4.1.3. Plant toxicity

Toxicity induced by FA is better elucidated in plant models. Fusarium wilt is caused when the xylem of host plants is infected with the fungus *Fusarium oxysporum*, a known producer of FA. This mycotoxin accelerated the development of wilt disease by damaging cell membranes of leaves and causing non-stomatal water loss (Wang et al., 2015). A study by Wu et al (2008) showed that FA inhibited photosynthesis through reduction in chlorophyll mass and caused leaf wilting and mass necrosis (Wu et al., 2008). Fusaric acid has been shown to affect the development of corn seedlings by inhibiting root growth through interfering with metabolic processes, since root hair elongation is an ATP dependent process (Diniz and Oliveira, 2009).

Fusaric acid has the ability to change the proton electrochemical gradient across the plasma membrane, increase the loss of electrolytes, decrease cellular ATP levels, and inhibit some metalloenzymes (e.g., cytochrome oxidase) leading to respiratory impairment. Experiments by Singh and Upadhyay (2014) have shown that FA induced cell death is through the associated increase in ROS production and lipid peroxidation and the down-regulation of anti-oxidative enzymes (Singh and Upadhyay, 2014). Jiao et al (2014) demonstrated that mitochondrial dysfunction is a crucial event in the programmed cell death of tobacco suspension cells. Cells treated with FA exhibited a decrease in mitochondrial membrane potential, ATP content and anti-oxidant activities as well as increased hydrogen peroxide (H₂O₂) and lipid peroxidation (LPO) (Jiao et al., 2014).

1.4.2. Pharmacology

Fusaric acid has also been shown to possess pharmacological activities that are apparent in the nervous and cardiovascular systems.

By partially inhibiting tyrosine hydroxylase and inhibition of dopamine-β-hydroxylase (DBH) FA is able to affect neurotransmitter levels in the brain contributing to the toxicity of this mycotoxin (Wang and Ng, 1999). Dopamine-β-hydroxylase is required for synthesis of norepinephrine in the brain. Smith and MacDonald (1991) observed changes to swine neurochemistry after acute doses of FA. Their key findings included elevated levels of brain tryptophan, serotonin, and 5-hydroxyindole acetic acid as well as behavioural changes such as refusal to feed and lethargy. They attributed lethargy to enhanced action of the serotonergic nervous system resulting from elevated blood and brain tryptophan concentrations and the subsequent synthesis of serotonin, triggering the onset of sleep (Smith and MacDonald, 1991).

Fusaric acid induced a reduction in blood pressure. A calcium salt of FA was tested in hypertensive patients for long term effects. In the first year both the systolic and diastolic blood pressures were lowered and no adverse effects were apparent. There were no consistent changes in heart rate or plasma volume. It was concluded that the hypotensive response was attained by reduction of the total peripheral vascular resistance index (Wang and Ng, 1999).

More recently studies have shown FA to possess tumoricidal potential. *In vitro* studies on the HL-60 cell line revealed that FA induced apoptosis and DNA fragmentation. Although both PA and FA induced apoptosis in this study, FA was seen to effectively initiate apoptosis at lower concentrations compared to its parent compound. Niacin related compounds have low permeability through the cell membranes due to their electrical charge and structure, therefore

the addition of a fat soluble side chain to the pyridine ring may make the compound more effective (Ogata et al., 2001).

Work by Stack JR et al (2004) elucidated a novel mechanism to treat head and neck squamous cell cancer (HNSCC). Fusaric acid can chelate divalent cations, especially zinc, and inactivate zinc finger proteins involved in DNA repair and protein synthesis. Their data demonstrated a suppressive effect of FA on two HNSCC cell lines. Fusaric acid reduced cell populations and arrested cell cycle progression. Although the exact mechanism is unknown FA is thought to cause DNA damage and inhibit DNA synthesis and repair. This may be brought on in part by its ability to chelate divalent cations from metalloproteins such as metallopeptidase-1, which is a zinc finger protein (Stack et al., 2004). Many metalloproteins are involved in DNA repair and promotion of on-going cell growth and proliferation, chelation may be an alternate means of inducing cell cycle arrest or apoptosis.

Ruda et al (2006) demonstrated that oral administration of FA inhibits tumour growth in an animal model for HNSCC. Following tumour cell inoculation mice were treated with FA or saline. The results of their study showed that FA treated mice had significantly slower tumour growth rates as well as decreased tumour mass when compared to control mice. This suggests a suppressive effect of FA on HNSCC xenografts (Ruda et al., 2006). The bioavailability of FA was determined by Stack et al., (2014) using male Sprague-Dawley rats. These investigators showed that 58% of the administered FA was available after oral administration suggesting that FA may be a viable oral therapeutic, however their study also revealed non-linear pharmacokinetic behaviour after administration of FA intravenously suggesting that metabolic enzymes and protein-FA interactions along with cellular transporters become saturated at a dose range of 10–75 mg/kg (Stack et al., 2014).

1.5. Mitochondria

Mitochondria are double membrane organelles and are sometimes described as “cellular power plants” due to their ability to convert organic material into energy (Kakkar and Singh, 2007). These organelles are responsible for the bulk of ATP production through various means including the Krebs’s cycle, β -oxidation of fatty acids and oxidative phosphorylation (OXPHOS) (Wang et al., 2013). They also play key roles in NADPH synthesis, DNA repair and metabolic pathways as well as playing a major role in cell death (Kakkar and Singh, 2007). Mitochondria are also the major producers of endogenous ROS in human cells, serving as „redox messengers“ regulating intracellular signaling (Wang et al., 2013).

Mitochondria contain four separate compartments, an inner membrane, an outer membrane, a matrix space enclosed by the inner membrane and an inter-membrane space between the inner and outer membranes. The Krebs's cycle occurs in the matrix of the mitochondria and the production of ATP occurs at the inner membrane (Champe et al., 2005).

The Krebs's cycle participates in several mitochondrial processes. It is the final pathway where oxidative metabolism of macromolecules (carbohydrates, protein and fatty acids) converge to produce the reducing equivalents NADH and flavin adenine dinucleotide (FADH₂), these reducing equivalents feed into the ETC (Champe et al., 2005).

Production of ATP occurs via the ETC. This chain is divided into multi-enzyme complexes that are embedded in the inter-mitochondrial membrane. They are complex I: NADH CoQ reductase, complex II: succinate-CoQ reductase, complex III: reduced CoQ cytochrome c reductase, complex IV: cytochrome c oxidase and complex V: ATP synthase which is made up of the F₁ and F₀ units (Kakkar and Singh, 2007).

The ETC is used to generate an electrochemical proton gradient across the inner membrane which is required for OXPHOS. Complex 1 (NADH hydrogenase) oxidizes NADH to NAD⁺ and initiates the process of electron flow. Electrons flow sequentially through complex III, cytochrome c and finally through complex IV where bound oxygen is reduced to water. During the process of electron flow from NADH to molecular oxygen, each of the three complexes (I, III, and IV) catalyzes the translocation of protons across the mitochondrial inner membrane. During OXPHOS the F₀ sub-unit transmits energy from the electrochemical proton gradient to the F₁ unit promoting ATP synthesis. This gradient facilitates the entry of adenosine diphosphate (ADP) and inorganic phosphate (Pi) into the matrix space. The ATP synthase complex then binds ADP to Pi producing ATP (Figure 1.8). The remaining part of the gradient is used to generate NADPH through a transmembrane enzyme called transhydrogenase, which is not part of the OXPHOS apparatus (Kakkar and Singh, 2007).

Iron-sulphur clusters and heme are iron containing prosthetic groups that catalyse the transfer of electrons in a variety of electrochemical reactions including those of the ETC. Mitochondrial ETC complex I, contains iron-sulphur clusters whereas complex II and III contain both heme and iron-sulphur clusters. Heme groups can also be found in cytochrome c and complex IV. Heme and iron-sulphur clusters only become biologically active when they are linked to a polypeptide backbone, which then adopts correct folding and conformation (Atamna et al., 2002). Aconitase is a crucial enzyme of the Krebs's cycle and is responsible for the inter-conversion of citrate and isocitrate. This enzyme contains iron-sulphur clusters and is highly

susceptible to oxidative inactivation lending itself to play a vital role as a mitochondrial redox sensor (Liu and Kamp, 2011). Thus both heme and iron-sulphur clusters are essential for the proper functioning of mitochondria.

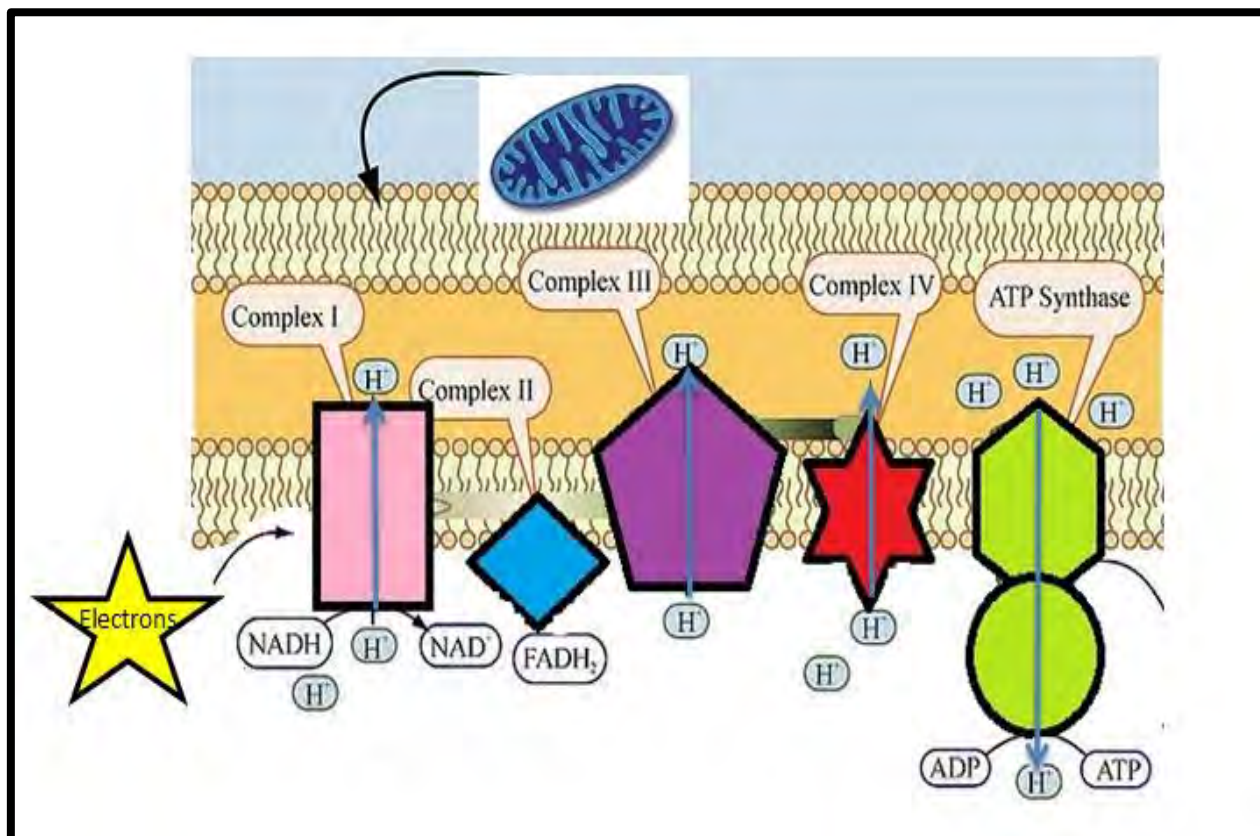


Figure 1.8: The electron transport chain is a sequence of protein complexes through which electrons flow in a unidirectional manner from complex I to complex V culminating in the synthesis of ATP (prepared by author).

1.5.2. Mitochondrial biogenesis

Mitochondrial biogenesis is defined as the growth and division of pre-existing mitochondria (Jornayvaz and Shulman, 2010) since they cannot be generated *de novo* (Battersby and Richter, 2013). Mitochondria are tubular in shape and changes to morphology are driven by fission, fusion and translocation. These processes allow for the proper organisation of the mitochondrial network during biogenesis (Ventura-Clapier et al., 2008). Mitochondrial fission is essential for the equal distribution of mitochondria in daughter cells during mitosis (Boland et al., 2013).

These organelles possess their own genome and can auto-replicate (Jornayvaz and Shulman, 2010). Mitochondrial DNA (mtDNA) is circular and encodes 13 mRNAs, 22 tRNAs and 2 rRNAs. All 13 mRNAs encode proteins necessary for OXPHOS. The remaining mitochondrial

proteins including those involved in mitochondrial biogenesis itself, are encoded by the nuclear genome (Yoboue and Devin, 2012). The co-ordinated synthesis and import of proteins encoded by the nuclear genome is required for correct mitochondrial biogenesis (Jornayvaz and Shulman, 2010). Because a major portion of mitochondrial proteins are encoded by nDNA (Figure 1.9), a variety of mechanisms exist to target, import, and correctly assemble these proteins thereby ensuring proper mitochondrial function and morphology (Ventura-Clapier et al., 2008).

Complex:	I	II	III	IV	ATP synthase
mtDNA:	7	0	1	3	2
nDNA:	38	4	10	10	14

Figure 1.9: The number of complex subunits encoded by mitochondrial (mtDNA) and nuclear (nDNA) genomes (Yoboue and Devin, 2012).

1.5.2.1. Transcription factors involved in mitochondrial biogenesis

The replication and transcription of mtDNA is driven by mitochondrial transcription factor A (Tfam) (Wu et al., 1999). This is a nuclear encoded transcription factor that binds to an upstream enhancer of the promoter sites of the two mitochondrial DNA strands (Ventura-Clapier et al., 2008). Tfam helps regulate mtDNA number (Piantadosi and Suliman, 2006) and Tfam expression is co-ordinated and regulated by a highly specific set of transcription factors mentioned below.

The nuclear respiratory factors (NRF-1 and NRF-2) are nuclear transcription factors that direct respiratory gene expression in cells. Both NRF-1 and NRF-2 are regulators for subunits of complex I, complex II, complex III, cytochrome oxidase and ATP synthase. These transcription factors also encode proteins involved in mtDNA transcription and replication and genes required for mitochondrial protein import (Yoboue and Devin, 2012).

The peroxisome proliferator-activated receptor gamma co-activator (PGC-1) family of co-activators (PGC-1 α , PGC-1 β and PRC) are highly versatile and have the ability to interact with a plethora of transcription factors allowing them to mediate a host of biological programs (Lin et al., 2005). Indeed, PGC-1 α plays a pivotal role in mitochondrial biogenesis by co-regulating other transcription factors (Scarpulla et al., 2012). PGC-1 α has emerged as the master regulator of mitochondrial biogenesis and respiration (St-Pierre et al., 2006). This co-activator mediates

the activity of several transcription factors involved in mitochondrial biogenesis by binding to transcription factors NRF-1, NRF-2 and estrogen-related receptor α (ERR α) among others (Yoboue and Devin, 2012). Initially, the NRF-1 transcription factor was identified as a target for PGC-1 α induced mitochondrial biogenesis. PGC-1 α can trans-activate NRF-1 mediated gene expression. PGC-1 α also targets ERR α along with NRF-2 in regulating respiratory genes such as cytochrome c. Therefore NRF-1 and NRF-2 are thought to act downstream from both PGC-1 α and ERR α in facilitating respiratory gene expression (Scarpulla, 2011).

The cyclic AMP (cAMP) response binding element protein (CREB) is a transcription factor that promotes the transcription of cAMP response element (CRE) regulated genes in response to different cellular signals. Phosphorylation of CREB by cAMP-dependent protein kinase (protein kinase A; PKA), as well as by Ca²⁺-dependent and other protein kinases, is required for transcriptional activation of CREB. De Rasmio et al., (2009) showed that CREB is imported into the mitochondria by a membrane potential dependent mechanism and this import may be facilitated by heat shock protein 70 (HSP70). Once in the mitochondria, phosphorylated CREB (p-CREB) promotes protein synthesis of mitochondrial OXPHOS subunits by exerting its effects on the mtDNA (Rasmio et al., 2009).

1.5.2.2. Integration of mitochondrial biogenesis and oxidative stress

Recent evidence shows a link between mitochondrial biogenesis and oxidative/mitochondrial stress responses. Oxidative stress is defined as the presence of ROS in excess of the anti-oxidant buffering capacity (Amira, 2010). Oxidative stress damages mitochondria impairing their ability to produce ATP ultimately leading to cell death. Many cellular programmes exist to protect mitochondrial integrity and to replace dysfunctional mitochondria with better suited organelles through replication of highly functional sub-populations (Piantadosi and Suliman, 2012).

Insight into the underlying mechanism of interactions between ROS signalling and mitochondrial biogenesis were elucidated by Piantadosi and Suliman (2006). Their study showed that lipid hydroperoxide regulates Tfam expression via AKT activation and its phosphorylation of NRF-1. This promotes the translocation of NRF-1 and binding to the Tfam promoter (Piantadosi and Suliman, 2006). Furthermore the induction of PGC-1 α expression is mediated by CREB binding to the PGC-1 α promoter during oxidative stress conditions (St-Pierre et al., 2006) further enhancing mitochondrial biogenesis. These results show that a link exists between the ROS producer (mitochondria) and its biogenesis.

Mitochondrial biogenesis can be activated by mitochondrial ROS via retrograde signalling from damaged mitochondria to the nucleus. These stress cues lead to up-regulation of nuclear transcriptional responses and subsequent encoding of mitochondrial and anti-oxidant proteins. The eliciting of such responses serve to compensate for mitochondrial dysfunction and dampen oxidative stress (Piantadosi and Suliman, 2012).

1.5.3. Mitochondrial stress responses

1.5.3.1. Sirtuins

The sirtuins belong to a conserved family of proteins that depend on NAD⁺ for their deacetylase activity. Although sirtuins are considered as histone deacetylases (HDACS) because of their NAD⁺ dependence, they are functionally different from other HDACS as they carry out deacetylation via a two-step reaction that encompasses the consumption of NAD⁺ and release of nicotinamide (NAM), 1-O-acetyl-ADP-ribose (1-OAADPR), and the deacetylated substrate. These deacetylases are involved in a range of biological functions including DNA repair, control of metabolic enzymes and apoptosis (Parihar et al., 2015).

In mammalian cells seven sirtuins exist (SIRT1-SIRT7), with each having distinct flanking C and N terminal extensions. These variations allow for sub-cellular localisation of SIRTs as described in table 1.

Table 1: The members of the Sirtuin family are found in different cell compartments allowing them to perform specific roles (Parihar et al., 2015)

<u>Sirtuin</u>	<u>Sub-cellular localisation</u>	<u>Substrates</u>	<u>Functions</u>
SIRT1	Nucleus	p53, Foxo1, Foxo3	Energy metabolism, stress response
SIRT2	Cytoplasm	Tubulin, H4, Foxo3a	Cell cycle
SIRT3	Mitochondria	Acetyl CoA synthase, Succinate dehydrogenase, Lon protease, SOD2	Energy metabolism, Stress responses
SIRT4	Mitochondria	Glutamate dehydrogenase	Amino acid metabolism
SIRT5	Mitochondria	Carbamoyl-phosphate synthase 1	Urea cycle
SIRT6	Nucleus	Histones	DNA repair
SIRT7	Nucleus	P53	rDNA transcription

1.5.3.2. Mitochondrial sirtuins

Mitochondrial sirtuins act as energy sensors due to their dependence on NAD^+ as a co-factor. The three mitochondrial sirtuins: SIRT3, SIRT4, and SIRT5 are localized mainly in the mitochondrial matrix due to the presence of a mitochondrion targeting sequences in their N termini. Among these SIRT3 is the major mitochondrial deacetylase that regulates global mitochondrial lysine acetylation status (Lombard et al., 2007) and maintains the integrity of this organelle (Tao et al., 2015). Initially the full length SIRT3 (44 kDa) is synthesized as an enzymatically inactive protein which is activated on its translocation to the mitochondria as a 28 kDa protein by a mitochondrial peptidase present in mitochondrial matrix. Once in the mitochondria SIRT3 regulates many important processes and is thus highly expressed in metabolically active organs such as the liver, brain and kidney (Parihar et al., 2015).

1.5.3.3. Sirtuin structure

All sirtuins contain a highly conserved catalytic core domain which has a structurally homologous and large NAD^+/NADH binding Rossmann-fold domain, zinc-binding domain and numerous loops that form a distinct and extended cleft. This cleft attaches the two domains where the NAD^+ and acetyl lysine containing protein substrates enter and bind to the enzyme for deacetylation (Figure 1.10) (Nogueiras et al., 2012).

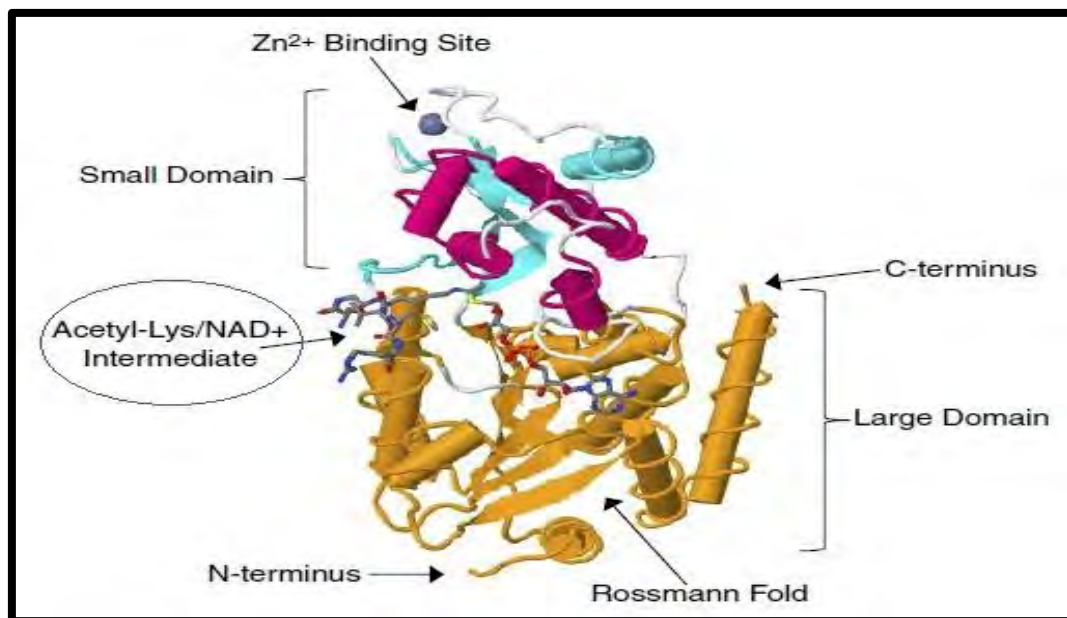


Figure 1.10: Structure of SIRT3 in complex with a substrate (encircled in blue)
(Nogueiras et al., 2012)

1.5.3.4. Regulation of SIRT3 activity

The expression of SIRT3 is dependent on the oxidative status of the cell, with stress conditions leading to the up-regulation of this mitochondrial protein (Weir et al., 2013). Mammalian sirtuins are not only regulated by NAD/NADH ratio or cellular stressors, but also by endogenous proteins involved in signal transduction and transcription, as well as by a number of microRNAs (Nogueiras et al., 2012).

The co-factor NAD^+ is an absolute requirement for the deacetylase activity of SIRT3. This places SIRT3 at the nexus of energy metabolism in the mitochondria (Nogueiras et al., 2012). The NAD^+ derived within a cell is via two main pathways: *de novo* synthesis from the amino acid tryptophan and via the salvage of NAM back to NAD^+ (Grant et al., 2009, Revollo et al., 2004). In humans, the rate-limiting step of the NAD salvage pathway is catalysed by nicotinamide phosphoribosyltransferase (NAMPT), which converts nicotinamide to nicotinamide mononucleotide (NMN) (Nogueiras et al., 2012). This reaction is followed by an NMN adenylyltransferase (NMNAT) dependent formation of NAD^+ (Figure 1.11) (Kleszcz et al., 2015). A study by Revollo et al., (2004) showed that NAD biosynthesis mediated by NAMPT, regulates the function of sirtuins and plays an important role in controlling various biological events in mammals (Revollo et al., 2004).

Sirtuins are also regulated by their reaction product, NAM, which inhibits sirtuin reactions through a base exchange pathway where rebinding of the reaction product to the enzyme accelerates the reverse reaction (Guan et al., 2014). However, through experimental kinetic and computational studies Guan et al., (2014) showed that NAM inhibition of SIRT3 involved competition between the inhibitor and enzyme co-factor NAD^+ (Figure 1.11), conflicting to the traditional characterization of base exchange as non-competitive inhibition (Guan et al., 2014).

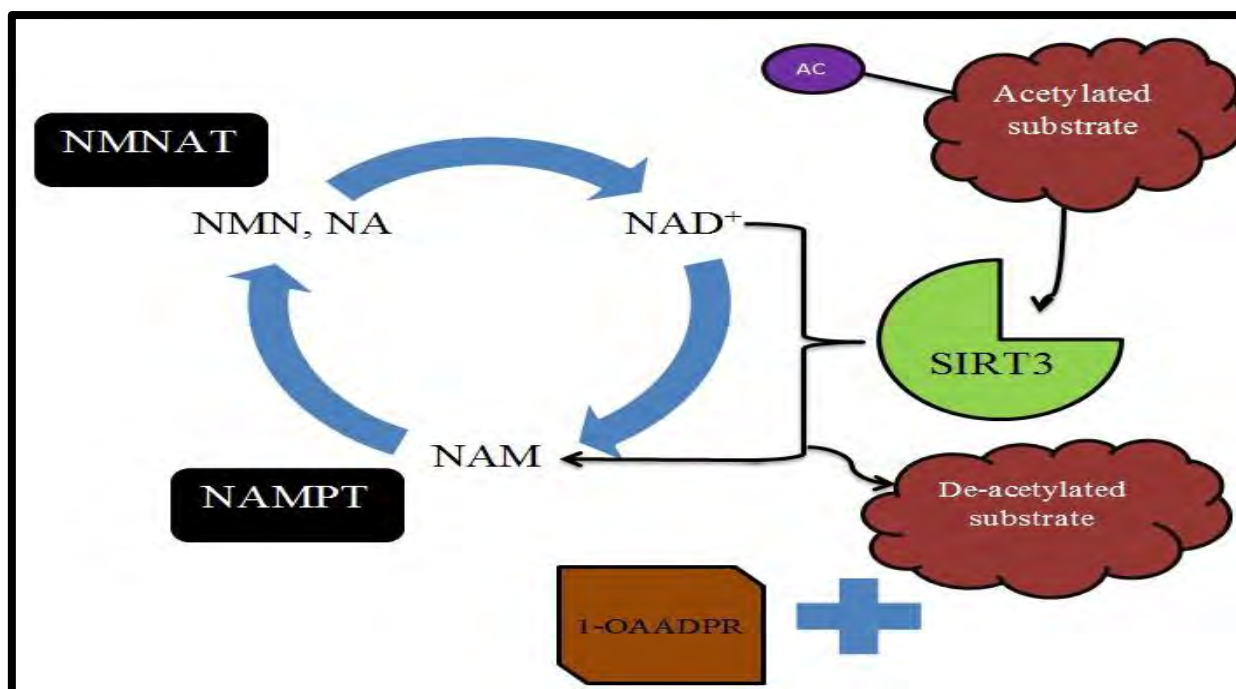


Figure 1.11: SIRT3 reaction and regulation (prepared by author).

1.5.4. Mitochondrial function is regulated by SIRT3

The mitochondria are a hub of metabolic activity for carbohydrate, protein and fatty acid processing. During metabolic activities most of the mitochondrial proteins undergo post-translational modifications with lysine acetylation serving as a link between acetyl CoA metabolism and cell signalling (Parihar et al., 2015). Indeed a vast majority of mitochondrial proteins are found to be acetylated (Gibellini et al., 2014a). Sirtuins, and in particular SIRT3 is therefore well positioned to regulate many aspects of mitochondrial function through removal of acetyl CoA from lysine residues through its deacetylase activity.

1.5.4.1. Fatty acid metabolism

The balance between fatty acid synthesis, lipid oxidation and its storage are the processes involved in fatty acid metabolism. Acetyl CoA synthetase is a substrate for SIRT3 (Tao et al., 2015). Schwer et al (2006) reported that human acetyl-CoA synthetase 2 (AceCS2) is a mitochondrial matrix protein that can be reversibly acetylated at Lys-642. The mitochondrial SIRT3 interacts with AceCS2 and deacetylates Lys-642 both *in vitro* and *in vivo*, thereby SIRT3 regulates fatty acid synthesis by activating the acetyl-CoA synthetase activity of AceCS2. The acetyl-CoA synthetase reaction, results in the formation of acetyl-CoA from acetate, ATP, and CoA, proceeds in two steps. In the first step, acetate is activated to acetyl-adenosine monophosphate (acetyl- AMP). In the second step, acetyl-AMP is converted to acetyl-CoA by the thioester bond-forming activity of ACS, and acetyl-CoA and AMP are released sequentially (Schwer et al., 2006).

Fatty acid oxidation is enhanced by SIRT3 deacetylation of long-chain acyl-CoA dehydrogenase (LCAD) which is generally hyperacetylated at one lysine residue, K42 (Parihar et al., 2015) and mitochondrial 3-hydroxy3-methylglutaryl CoA synthase 2 (HMGCS2), which catalyses the process of lipid utilization (Shimazu et al., 2010). This deacetylase also prevents the accumulation of fatty acids in the mitochondria by acting alongside the adenosine monophosphate activated protein kinase (AMPK), which phosphorylates acetyl CoA carboxylase (ACC) the rate limiting enzyme in fatty acid synthesis (Shi et al., 2010).

1.5.4.2. Kreb's cycle, Electron transport chain and oxidative phosphorylation

The protein complexes of the ETC and OXPHOS are the most heavily acetylated. Since SIRT3 is the major mitochondrial deacetylase it interacts with proteins of the ETC, modifying and improving their activity (Parihar et al., 2015). The activity of complexes I and II of the ETC was significantly decreased in SIRT3 knock-out mouse embryonic fibroblasts (MEFs). This study also eluded to a role of SIRT3 in complex III regulation (Kim et al., 2010). Complex V of the ETC was shown to be a target of SIRT3 with deacetylation of this complex increasing its activity (Finley et al., 2011). The importance of SIRT3 on energy homeostasis was demonstrated by Ahn et al (2008). They showed that the knock-out of SIRT3 resulted in a 30% decrease to intracellular ATP levels (Ahn et al., 2008).

A study by Finley et al., (2011) showed that SIRT3 directly interacts with succinate dehydrogenase (SDH) sub-units A and B. The SIRT3 mediated deacetylation of this complex serves to increase enzyme activity. The SDH complex is a member of both the ETC and Kreb's cycle (Finley et al., 2011), this shows that SIRT3 is a regulator of FAD/FADH₂ metabolism. The efficient deacetylation of the Kreb's cycle enzyme isocitrate dehydrogenase (ICDH) was observed by Schlicker et al., (2008). Their study revealed that SIRT3 interacts with ICDH and increased its activity through deacetylation of lysine211 or 212 (Schlicker et al., 2008).

1.5.4.3. Integration of oxidative stress and mitochondrial stress responses

The primary defence against oxidative stress is the detoxifying enzymes that scavenge excess ROS. These include catalase and superoxide dismutase. The role of PGC-1 α in ROS metabolism was explored by St-Pierre et al., (2006). They reported that the expression of mitochondrial ROS-detoxifying enzymes including GPx1 and SOD2 increases with PGC-1 α . They showed that by increasing PGC-1 α levels neural cells in culture were protected from oxidative stressor-mediated death. These studies revealed PGC-1 α 's role as a broad and powerful regulator of ROS metabolism (St-Pierre et al., 2006).

Recent evidence has shown that PGC-1 α links mitochondrial stress responses to oxidative stress. Oxidative stress increases the expression of PGC-1 α , which induces SIRT3 expression through activation of ERR- α . In turn, SIRT3 stimulates PGC-1 α expression *via* pCREB, thereby forming a positive feedback loop and increasing anti-oxidant defence (Kong et al., 2010).

In the mitochondria SIRT3 is able to deacetylate and increase the activity of SOD2. Two recent studies (Tao et al., 2010, Qiu et al., 2010) indicated that SOD2, the primary mitochondrial superoxide detoxification enzyme, contains a lysine residue that can be deacetylated by SIRT3 overexpression. Deeper analysis by Tao and co-workers (Tao et al., 2010) showed that lysine 122 is directly deacetylated by SIRT3. When lysine 122 was altered to arginine (to mimic the deacetylated state; MnSODK^{122-R}), enzymatic activity was increased, intracellular ROS were decreased, and stress-induced genomic instability was prevented. In contrast, when lysine 122 was altered to a glutamine (to mimic the acetylated state; MnSODK^{122-Q}), SOD2 activity was decreased, proposing that acetylation status directs SOD2 enzymatic activity and cellular ROS levels (Tao et al., 2010). Increased ROS levels in SIRT3 knock-out mouse embryonic fibroblasts were also observed by Kim et al., (2010). Furthermore, SIRT3 was found to deacetylate FOXO3a and allows its translocation to the nucleus (Kim et al., 2010). The mammalian FOXO transcription factors are targeted by sirtuins under conditions of oxidative stress and determine their subcellular localisation, protein stability, and transcriptional activity leading to up-regulation of anti-oxidant expression such as catalase (Rajendran et al., 2011). Taken together the results of these studies outline a crucial role for SIRT3 in dampening oxidative stress.

Besides its roles as a regulator of ROS scavenging, SIRT3 plays an important role in maintaining mitochondrial structure and function through the deacetylation of Lon protease (LON). Gibellini et al., (2014) found that LON and SIRT3 co-localise and co-immunoprecipitate in breast cancer cells and SIRT3 loss increased the acetylation and protein expression of LON. Their study suggests that SIRT3 deacetylates LON most likely at lysine 917 (Gibellini et al., 2014a). LON is also regulated at the transcriptional level by Nrf2 (Ngo et al., 2013). This protease is a human stress protein that removes and degrades oxidatively damaged proteins in the mitochondria preventing their cross linking and aggregation. A study by Ngo and Davies (2009) showed that loss of LON leads to increased protein damage and mitochondrial dysfunction (Ngo and Davies, 2009).

The catalytic activity of LON is ATP dependent and serves to not only remove damaged proteins but regulate mitochondrial pathways by terminating or modulating the activity of

protein or protein complexes through selective sub-unit degradation (Venkatesh et al., 2012). The steps involved in proteolytic protein degradation by LON are depicted in Figure 1.12.

LON is a mitochondrial DNA binding protein that interacts with single stranded DNA only in mammalian cells. Human LON associates with mtDNA sequences bearing a minimum of 4 adjoining guanine residues that have a tendency to form G-quadruplexes, which are four-stranded intra- or inter-molecular structures with a tetrad organization of guanines. The binding of mtDNA and LON is a physiological function of this protease and may provide a mechanism for recruitment of it to sites in mtDNA where it can degrade or process proteins involved in mtDNA and mitochondrial RNA metabolism. Furthermore the binding of mtDNA by LON may directly promote or inhibit the processivity or timing of transcription and/or replication (Venkatesh et al., 2012). Matsushima et al., (2010) investigated the role LON protease plays in regulating mtDNA transcription and maintenance. Their results show that LON modulates biogenesis of mtDNA through selective degradation of Tfam. Thus stabilising the Tfam:mtDNA ratio by degradation of excess Tfam (Matsushimaa et al., 2010). Under oxidative stress conditions LON binds to fewer mtDNA sites. Decreased LON binding may occur due to DNA repair and replication proteins being recruited to the mitochondrial genome and competing with Lon for binding sites. Alternatively, Lon may be recruited away from the genome to degrade other proteins that become oxidativelydamaged. These results suggest roles for LON in linking protein and mtDNA quality control (Lu et al., 2007).

LON can also act as a chaperone, independent of its proteolytic activity and promote the assembly of cytochrome c oxidase subunits (Bota et al., 2005) with downregulation of LON resulting in impaired respiratory function. Gibellini and colleagues (2014) showed that decreased LON levels impairs mitochondrial function in colorectal cancer cells with concomitant decreased expression of mitochondrial proteins of which some were related to Kreb's cycle and respiration (Gibellini et al., 2014b).

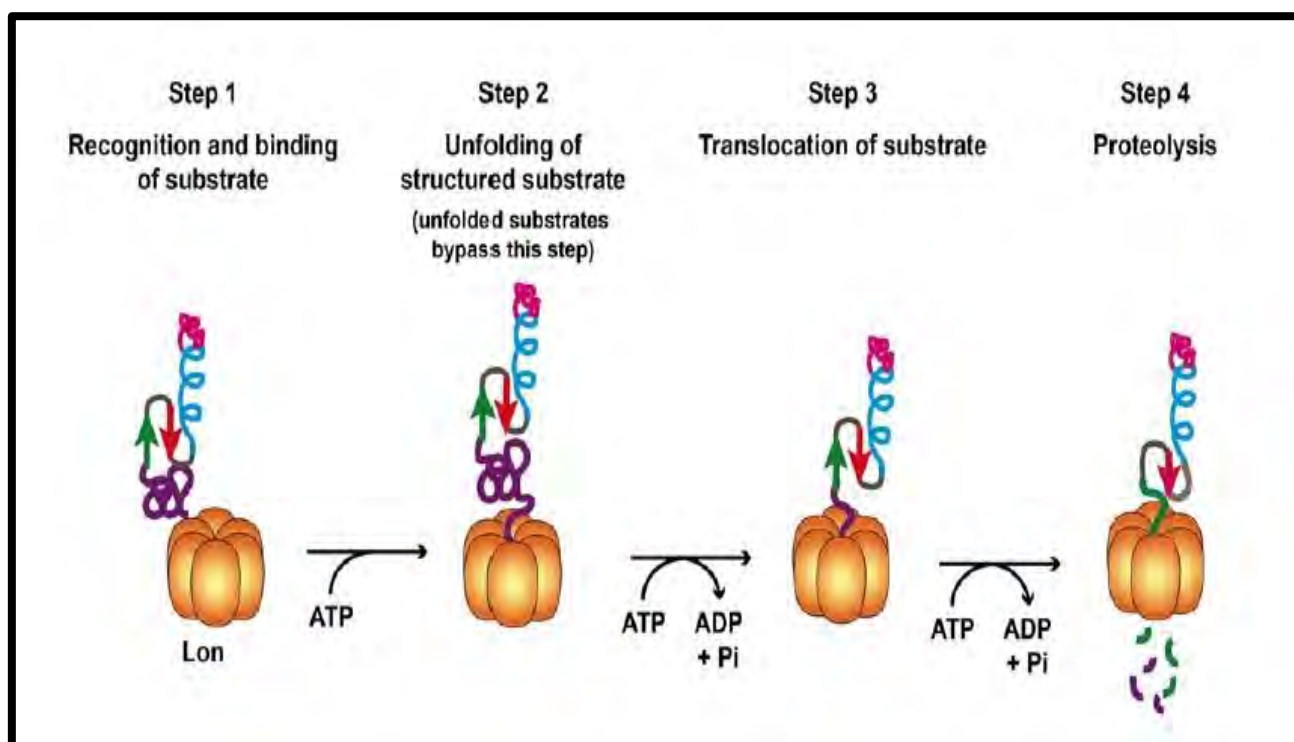


Figure 1.12: General mechanism for the recognition and degradation of protein substrates by LON. Step 1: recognition and binding of a sequence found in the protein substrate. Step 2: unfolding of structured protein substrates by the AAA⁺ domains of the holoenzyme, this requires ATP -binding and -hydrolysis. Unfolded substrates or peptides bypass this step. Step 3: translocation of unfolded polypeptides or short peptide sequences into the degradation chamber, which also requires ATP -binding and -hydrolysis. Step 4: peptide bond cleavage resulting in the generation of small peptide products. Continuous substrate unfolding and translocation are required to degrade substrates completely (Venkatesh et al., 2012).

1.6. Cellular responses to oxidative stress

Cells are able to sense macromolecular damage and respond to stress-induced damage, thereby re-establishing homeostasis. Following the initiation of oxidative stress, the basic leucine zipper transcription factor, nuclear factor-erythroid 2-related factor 2 (Nrf2) is activated at the posttranscriptional level. Nrf2 is the master transcription factor plays a pivotal role in cell defence against oxidative stress by modulating the anti-oxidant response programme (Keum and Choi, 2014). This prompts the expression of genes encoding proteins functioning as antio-

xidants and enzymes involved in phase II detoxification and glutathione biosynthesis (Dinkova-Kostova et al., 2005).

Under non-stressed conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). This complex directs Nrf2 polyubiquitination and degradation by functioning as an adaptor of the Cul3 based E3 ligase (Dinkova-Kostova et al., 2005). Upon oxidative stress, Nrf2 is liberated from Keap1 and enters the nucleus, to stimulate the expression of anti-oxidant response element (ARE) containing genes (Sano and Fukuda, 2008, Keum and Choi, 2014). This prevents oxidative damage to cellular components and organelles.

A study by Dinkova-Kostova et al. (2005) sought to determine how the kinetics and stoichiometry of Keap1 govern its susceptibility to inducer mediated modifications. Their results revealed that Keap1 contains 0.9 zinc atoms per monomer and established that zinc is bound to the reactive cysteine thiols of Keap1 and that inducers displace this metal. They also revealed that zinc binding is highly dependent on these cysteine residues and that mutation of these residues to alanine decreased binding by 2 fold. Thus, regulation of the phase II response involves chemical modification of critical cysteine residues of Keap1, whose reactivity is modulated by zinc binding. Keap1 is a zinc-thiol protein enabled with a switch controlled by both metal-binding and thiol reactivity. Under non-stressed conditions Keap1 binds zinc that is coordinated in part by reactive cysteine residues (Cys). In this conformation, Keap1 binds Nrf2 and marks the transcription factor for degradation. Upon inducer sensing, the zinc is released and the reactive cysteine residues are modified by alkylation, oxidation, or thiol-disulfide interchange, leading to a conformational change that separates the Kelch domains and releases Nrf2 (Figure 1.13) allowing its nuclear translocation and enhanced expression of phase 2 response genes (Dinkova-Kostova et al., 2005).

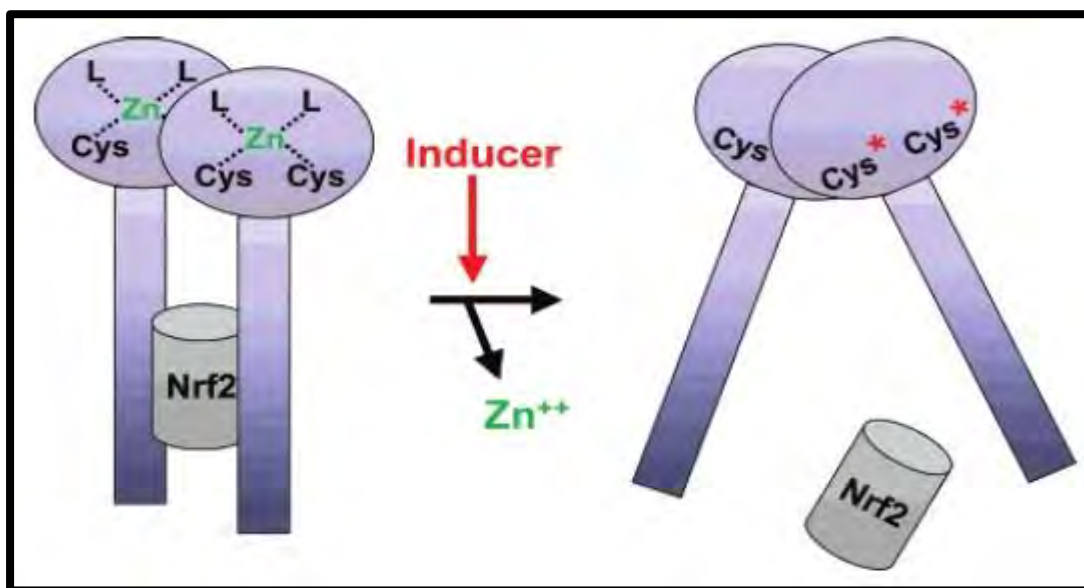


Figure 1.13: Role of zinc in mediating Nrf2 release (Dinkova-Kostova et al., 2005).

1.7. Mitochondria in hepatotoxicity

Damage to hepatocytes can be cytotoxic, genotoxic or metabolic. Cytotoxicity involves morphological changes to the hepatocyte often accompanied by the leakage of hepatic enzymes to the extracellular environment. Genotoxins cause DNA damage and often lead to the development of hepatocarcinomas. Finally, toxins are also able to alter cellular metabolism of hepatocytes and can lead to cell death (Castell et al., 1997).

Hepatotoxicity often originates from the alteration to metabolic processes within the cell. Some toxins directly inhibit enzyme activity or ion transport or compete with cellular metabolites for metabolic pathways in hepatocytes. Other toxins can affect the ATP status of the cell by increasing ATP consumption or decreasing ATP synthesis (Castell., 1997).

The mitochondria constitute an important target for hepato-toxins. Even a mild dysfunction of mitochondria in the liver can cause hepatic injury. Dysfunctional mitochondria lead to the excessive generation of ROS, this causes damage to cellular components including lipids, protein and nucleic acids (Esposti et al., 2012). Mitochondria play a pivotal role in apoptosis. This is an energy dependent form of programmed cell death. The mitochondria are the initiators of the intrinsic apoptotic programme which relies on stimulators opening the mitochondrial permeability transition (MPT) pore leading to the loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol. The first group consists of cytochrome c, second mitochondria-derived activator of caspases (Smac)/DIABLO, and the serine protease

HtrA2/Omi. These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates apoptosis protease activation factor (APAF-1) as well as procaspase-9, forming an “apoptosome”. The clustering of procaspase-9 in this manner leads to caspase-9 activation. Caspase 9 subsequently cleaves pro-caspase 3 leading to caspase 3 activation. Smac/DIABLO and HtrA2/Om are reported to promote apoptosis by inhibiting inhibitors of apoptosis proteins (IAP) activity (Elmore, 2007).

The IAP family of proteins shares a conserved module known as the baculovirus inhibitor of apoptosis protein repeat (BIR) domain. The BIR is a zinc finger protein characterized by the conservation of critical Cystine and Histidine residues that coordinate a zinc atom. A total of six major IAPs have been identified in humans, of which four directly inhibit the activity of caspases (Riedl et al., 2001).

Structural analysis of X-linked mammalian inhibitor of apoptosis protein (XIAP) reveals that the segment inhibiting caspase 3/7 is distinct from the segment inhibiting caspase 9 activity with the BIR2 and BIR3 domains associated with inhibiting the effector and initiator caspase respectively (Riedl et al., 2001).

The second group of pro-apoptotic proteins, apoptosis inducing factor (AIF) and endonuclease G are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell has committed to die. AIF translocates to the nucleus and causes DNA fragmentation and condensation of peripheral nuclear chromatin. Endonuclease G also translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments. AIF and endonuclease G both function in a caspase-independent manner (Elmore, 2007).

Dysfunction of this critical organelle affects energy balance and is a crucial factor in cell death (Castell., 1997). Hepatotoxicity studies most often deal with a form of cell death termed lytic necrosis. This form of cell death is characterised by membrane lysis and inflammation. (Castell., 1997).

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

The HepG2 cell line was purchased from Highveld Biologicals (Johannesburg, South Africa (SA)). All tissue culture reagents, the Caspase-Glo[®] 9 and 3/7 Assay and ATP assay (Promega) were obtained from Whitehead Scientific (Johannesburg, SA). Western blot reagents were purchased from Bio-Rad ((Hercules, CA, USA). Quantitative PCR (qPCR) primers were synthesized by Inqaba Biotech. All other reagents were purchased from Merck (Darmstadt, Germany).

2.2. Cell culture and exposure protocol

2.2.1. Cell culture

The HepG2 cell line is derived from a liver hepatocellular carcinoma of a 15 year old Caucasian male. These cells are adherent, non-tumorigenic and are epithelial in nature. There is no evidence of the HBV genome in this cell line (ATCC, 2014). This cell line is considered a model for the investigation of toxicity since it retains the ability to undergo detoxification responses as well as retaining many functions often lost by primary hepatocyte cultures (Mersch-Sundermann et al., 2004). The HepG2 cells were cultured in complete culture media (CCM) consisting of Eagle's minimum essential medium supplemented with 1% penstrepfungizone, 1% L-glutamine and 10% foetal bovine serum. Cultures were maintained at 37°C with 5% CO₂.

2.2.2. Exposure protocol

For the methyl thiazol tetrazolium (MTT) assay, cells were seeded into a 96-well microtitre plate, allowed to attach overnight and treated with FA solution (0-500µg/ml) for 24h. The range and time frame utilized is based on previous work (Jiao et al., 2014, Telles-Pupulin et al., 1998, Ruda et al., 2006). The chosen time period will provide a suitable measure for acute exposure. For all other assays cells were cultured to 90% confluency in 25cm³ tissue flasks and treated with FA at the half maximal inhibitory concentration (IC₅₀) as determined by the cell viability assay. The assays were performed at the IC₅₀ of cell viability since lower concentrations might result in cytotoxic effects being underestimated, while higher concentrations could result in experimental by-products. An IC₅₀ provides a workable concentration.

2.2.2.1. Use of controls

For western blot and q-PCR experiments a negative control and positive control was used. The negative control contained only CCM whereas the positive control was treated with 10 mM

nicotinamide. The significance for using the positive control was to show an expected outcome in relation to SIRT3 loss.

2.3. Metabolic activity

2.3.1. MTT assay

2.3.1.1 Introduction

The colorimetric MTT assay was used to determine cell viability. Methylthiazol tetrazolium is a yellow water soluble dye that is reduced by metabolically active cells through the activity of dehydrogenase enzymes. This leads to formation of an insoluble intracellular purple formazan product which can then solubilized and quantified by spectrophotometry.

This reaction only occurs in metabolically active cells and is dependent on the production of the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) produced in the Krebs's cycle (Figure 2.1). This assay is a measure of metabolic activity in the cell. The intensity of the formazan product is directly proportional to metabolic activity and cell viability (Figure 2.2).

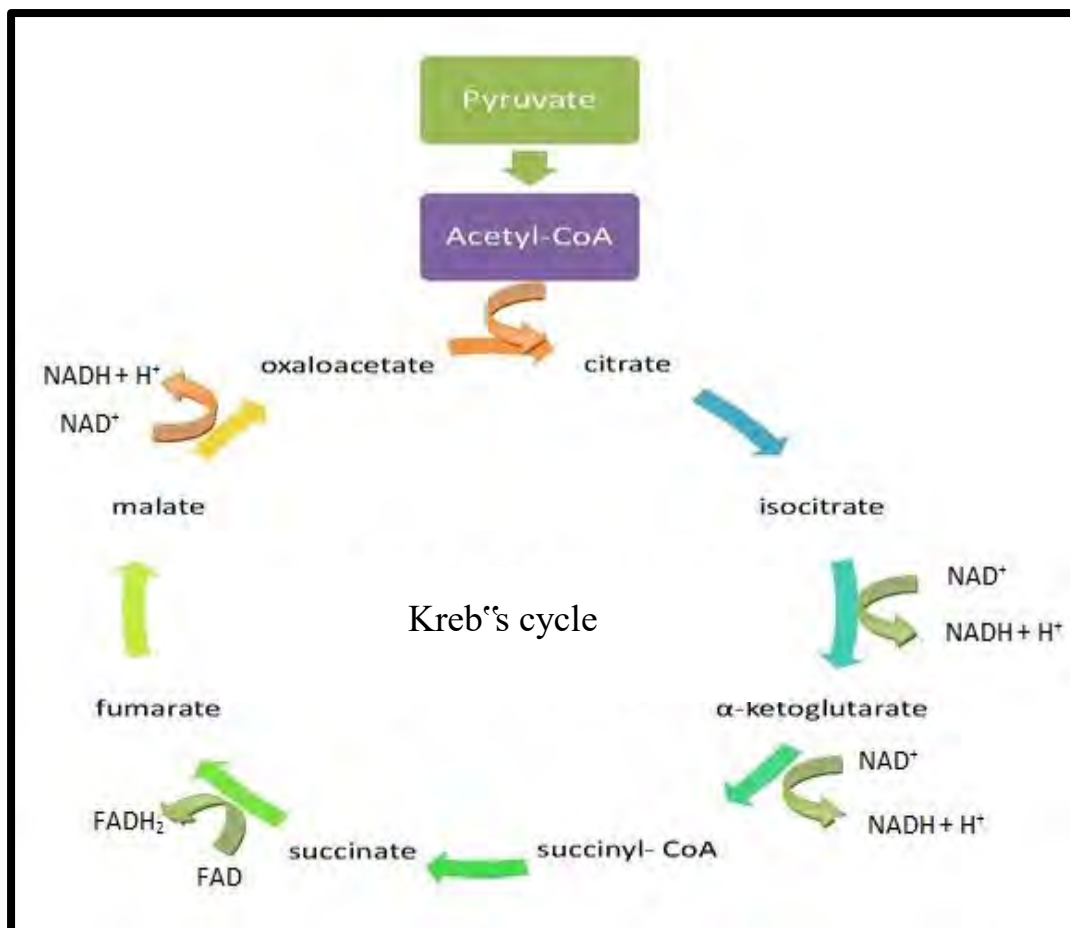


Figure 2.1: Production of reducing equivalents by the Krebs's cycle to drive the ETC and produce ATP (prepared by author).

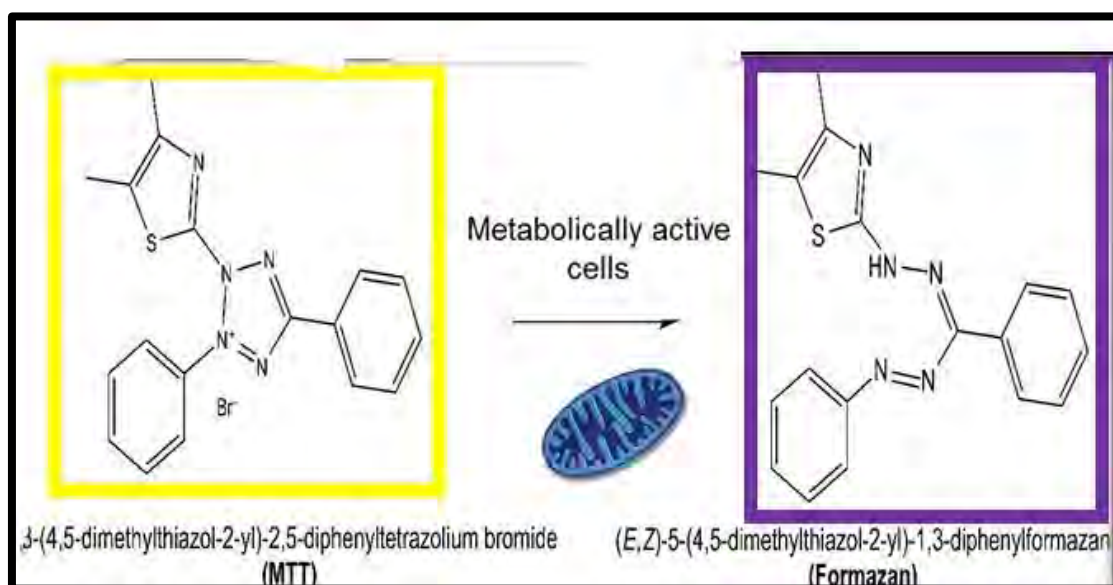


Figure 2.2: Schematic representation of the principle behind the MTT assay (prepared by author).

2.3.1.2. Protocol

Approximately 20,000 cells were seeded per well in triplicate and incubated with a range of FA concentrations (0, 25, 50, 100, 250, 350 and 500 µg/ml) for 24 h. Cells were then rinsed twice with 0.1M phosphate buffer saline (PBS) and incubated with MTT salt solution (5mg/ml in 0.1M PBS) and complete culture medium for 4h (37°C). Subsequently, 100µl of dimethyl sulphoxide (DMSO) was added to each well and incubated for 1h (37°C). The optical density of the formazan product was read using a spectrophotometer (Bio-tek µQuant) at 570 nm with a reference wavelength of 690 nm. The results were expressed as percentage cell viability vs. concentration of FA, from which the IC₅₀ was determined.

2.3.2. ATP assay

2.3.2.1. Introduction

Adenosine triphosphate is the major energy currency molecule of the cell and its production is essential to carry out various cellular processes. The mitochondria are the predominant producers of ATP through the electron transport chain coupled to oxidative phosphorylation as well as through substrate level phosphorylation. Glycolysis also contributes to ATP production under certain conditions. These processes ensure optimum production of ATP necessary for cell survival.

To determine ATP concentration, the CellTire Glo™ (Promega) assay was used. This assay employs bioluminescence to measure ATP levels in cells and is based on the luciferase reaction in which luciferin is mono-oxygenated to oxy-luciferin in the presence of Mg²⁺, molecular oxygen and ATP. This results in the release of energy in the form of luminescence (Figure 2.3). This luminescent signal is directly proportional to the ATP concentration in the cells.

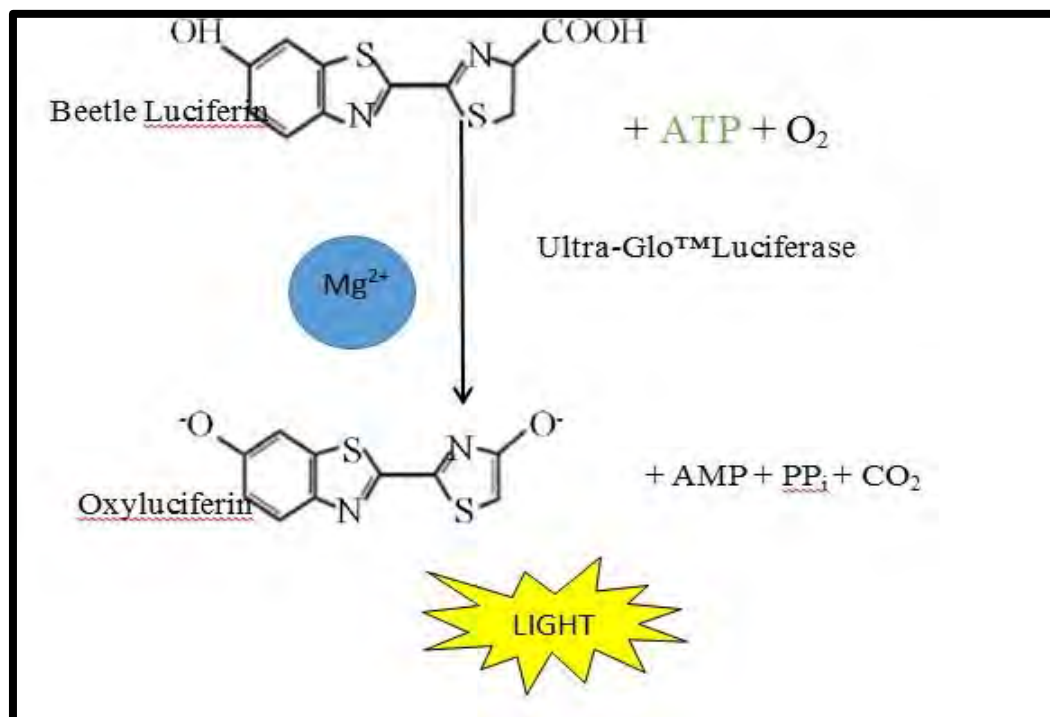


Figure 2.3: Principle of the CellTire Glo™ assay used to quantify intracellular ATP concentration (prepared by author).

2.3.2.2. Protocol

Approximately 20,000 cells were aliquoted per well in a microtitre plate (triplicate) to which 50 µl CellTire Glo™ reagent (Promega, Madison, USA) was added and left in dark (30 mins, RT) to allow reaction to occur. The luminescent signal was then read using a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The signal is directly proportional to intracellular ATP concentration. Results are expressed as mean relative light units (RLU).

2.4. Oxidative stress – Lipid peroxidation

2.4.1. Introduction

Lipid peroxidation is defined as the oxidative deterioration of lipids of which the unsaturated variety is most affected due to their double bond between carbon atoms. Many cellular organelles incorporate lipids into their membranes, thus damage caused by peroxidation is detrimental to cell function and survival (Devasagayam et al., 2003).

Lipid peroxidation is initiated by oxidizing agents such as free radicals that remove hydrogen atoms from poly-unsaturated fatty acids (PUFAs). This brings about a chain reaction mechanism of lipid peroxidation which involves an initiation step, a propagation step and termination step (Ayala et al., 2014, Devasagayam et al., 2003).

The thiobarbituric acid reactive substances (TBARS) assay was used to quantify the concentration of malondialdehyde (MDA), a by-product of lipid peroxidation and marker of

oxidative stress. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) at high temperature and low pH resulting in formation of a pink chromagen (Figure 2.5). During initiation, oxidizing agents remove hydrogen atom forming a lipid radical; this radical can be stabilized by a molecular rearrangement to form a conjugated diene (step 1). In the propagation phase, lipid radical rapidly reacts with oxygen to form a lipid peroxy radical (step 2) which removes a hydrogen from another lipid molecule generating a new lipid radical (step 3). In the termination reaction, anti-oxidants donate a hydrogen atom to the lipid peroxy radical species resulting in the formation of nonradical products (step 4, Figure 2.4) (Ayala et al., 2014).

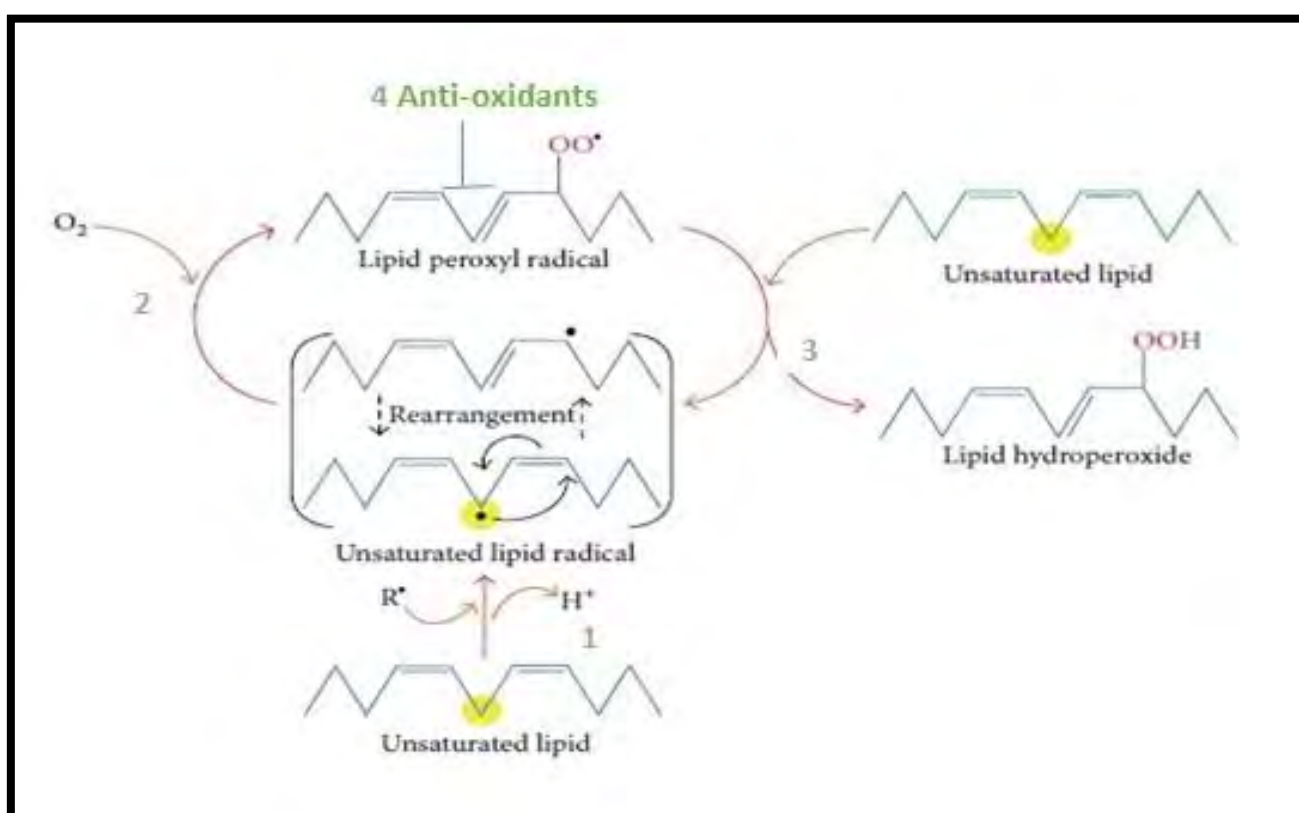


Figure 2.4: Chain reaction of lipid peroxidation. Step1 – initiation, step 2 and 3 – Propagation, step 4 – Termination (Ayala et al., 2014).

2.4.2. Protocol

The following reagents were added to a set of test tubes: 200 μ l of 2% H_3PO_4 , 400 μ l of 7% H_3PO_4 , 200 μ l of TBA/butylated hydroxytoluene (BHT) solution and 200 μ l of 1M HCL. Supernatants of treated cells were recovered and 100 μ l of cell supernatant was then added to each test tube in triplicate. A positive control was prepared by adding 1 μ l of MDA to a test tube. Samples were then boiled (100°C, 15min) and after cooling; butanol (1.5ml) was added to each

tube, vortexed for 10 secs and allowed to separate into two distinct phases. The upper butanol phase (100µl, triplicate) was then transferred to a 96-well micotitre plate and the absorbance read using a spectrophotometer (Bio-tek µQuant) at 532nm. The mean absorbance was divided by the extinction co-efficient (156mM^{-1}) and results were expressed as µM concentrations.

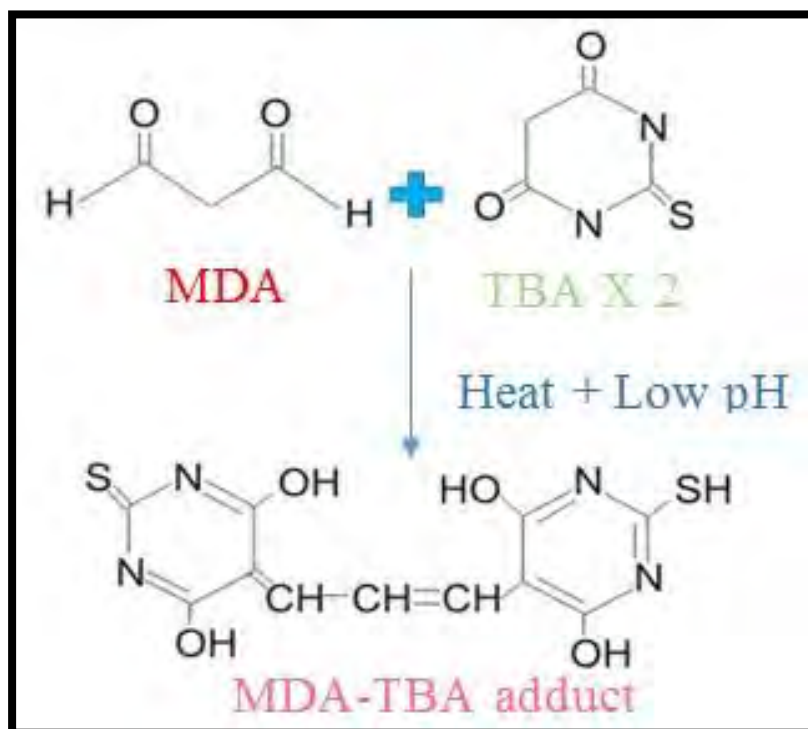


Figure 2.5: Schematic representation of the TBARS assay principle (prepared by author).

2.5. Protein expression – western blot

2.5.1. Introduction

Western blot is used to detect the presence of a protein of interest from a homogenous mixture of proteins extracted from cells. This procedure relies on the separation of proteins according to their size, transfer of these proteins to a solid support, detection of the protein of interest using the appropriate anti-body and visualisation using chemiluminescence.

2.5.1.1. Sample preparation

Sample preparation involves lysis of cells to release proteins and quantification. This processes are carried out on ice to prevent degradation of the protein of interest. The samples must be quantified and standardised to ensure that protein concentration is sufficient to carry out the assay and to compare protein expression in each sample. The bicinchoninic acid (BCA) assay is a commonly used method to quantify total protein in a sample. The principle of this assay relies on the formation of Cu^{2+} -protein complex under alkaline conditions and subsequent reduction of Cu^{2+} to Cu^{1+} . The protein present is directly proportional to this reduction. BCA forms a violet complex with Cu^{1+} under alkaline conditions therefore provides a basis to monitor the reduction of Cu^{2+} by proteins. The BCA-Cu^{2+} complex is a relatively stable chromophore that absorbs at 562 nm (Figure 2.5) (Sapan et al., 1999).

Once samples are standardised they are diluted in Laemmli buffer (contains glycerol - samples sink easily into the wells of the gel, bromophenol blue - a tracking dye which indicates how far the separation has progressed, β -mercaptoethanol – reduce disulphide bridges of proteins allowing them to unfold and SDS –neutralizes protein charges) so that they can be electrophoresed (Healthcare, 2011)

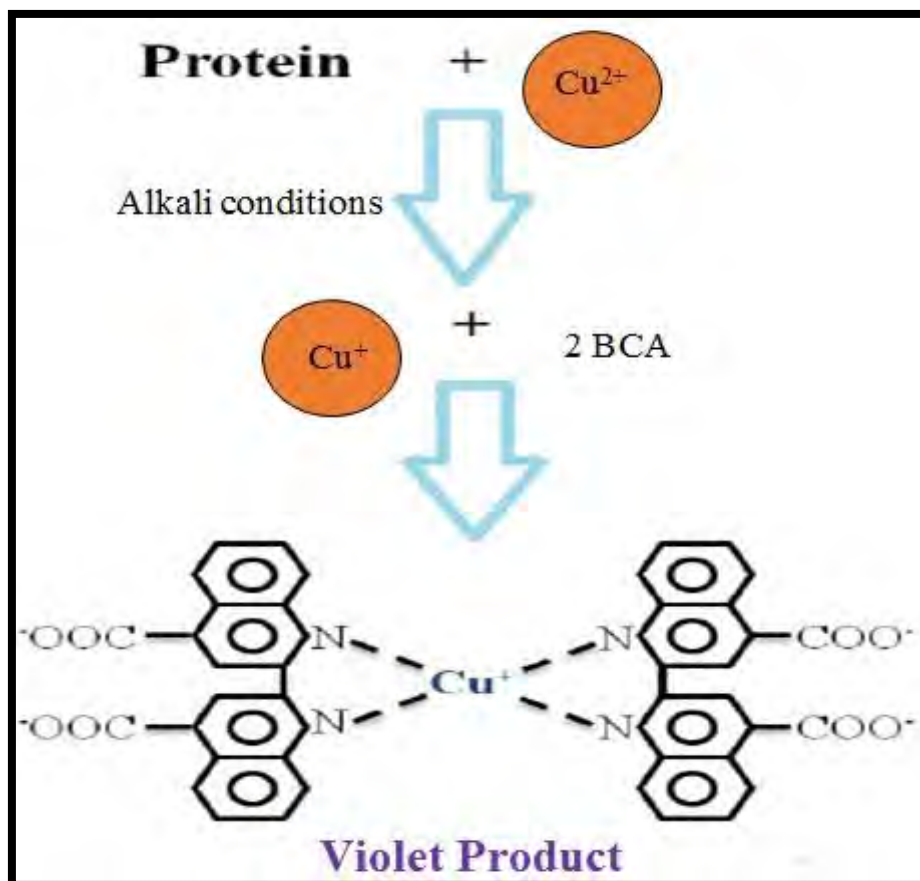


Figure 2.5: Principle of the BCA assay used to quantify protein concentration (prepared by author).

2.5.1.2. Separation of proteins

Proteins are separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Since samples are denatured by the Laemmli buffer containing SDS the protein is uniformly charged (negative charge) and migrates toward the positive electrode through the gel. Since both the charge and tertiary structure of the proteins have been removed the proteins separate according to size through the polyacrylamide gel (Figure 2.6) (Healthcare, 2011).

Polyacrylamide gels are inert with a cross-linked structure this enables it to retard the migration of larger molecules allowing smaller molecules to pass through the gel at a faster rate. The gel consists of two sections – resolving gel and stacking gel. The resolving gel is cast first and has a high concentration of acrylamide, this is important for separating proteins by size. The stacking gel is cast after the resolving gel has set and serves as a “start line” for protein migration (Healthcare, 2011)

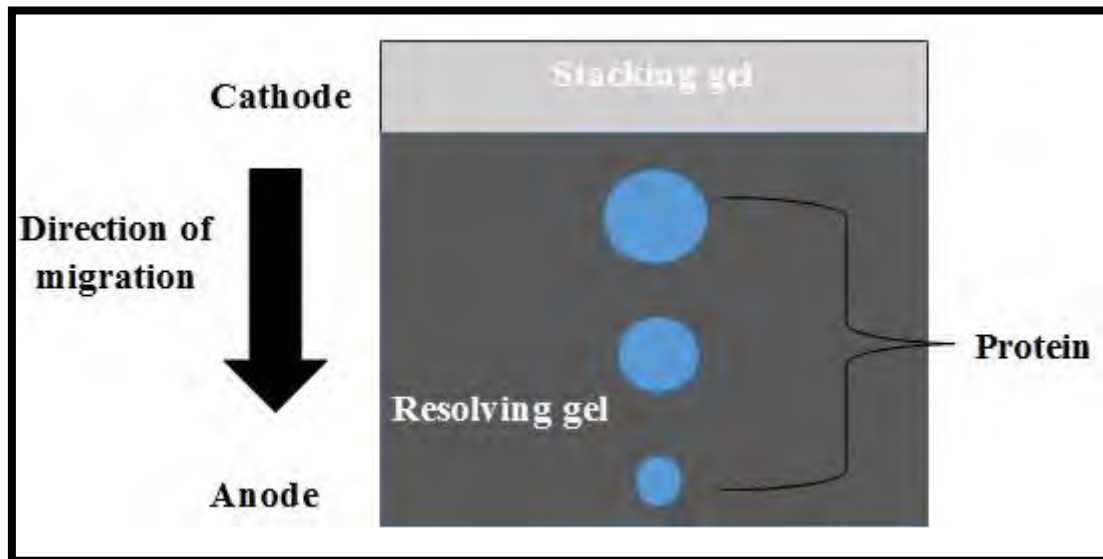


Figure 2.6: separation of proteins according to size (prepared by author).

2.5.1.3. Transfer of proteins to solid support

On completion of the separation of proteins, the next step is to transfer the proteins from the gel to a solid support membrane (Figure 2.7), usually made of an inert substance, like nitrocellulose. The proteins transferred from the gels are immobilized at their corresponding migratory positions at the time point when the electric current on the gel run was stopped (Healthcare, 2011).

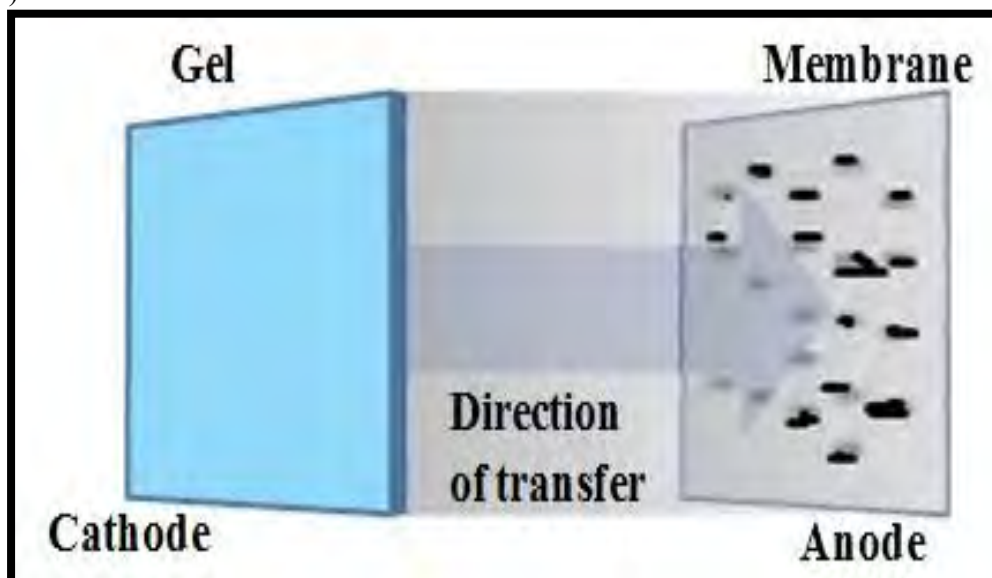


Figure 2.7: Transfer of proteins from gel to membrane. The gel is placed in contact with the membrane and the proteins migrate toward the positively charged anode in an electric field (prepared by author).

2.5.1.4. Immuno-blotting

After transfer the protein of interest is detected using a specific antibody. A non-labelled primary anti-body is directed against the protein of interest and a species specific, labelled secondary antibody is directed against the primary anti-body. This secondary anti-body serves as a carrier of the label and is involved in signal amplification, since in theory many secondary anti-bodies can simultaneously bind to a single primary anti-body. The secondary anti-body is conjugated with horseradish peroxidase (HRP). In the presence of H_2O_2 , HRP oxidises luminol to generate light (Figure 2.8). The intensity of the light emitted is directly proportional to the expression of the protein of interest. Detection reagent is used to amplify signals (Healthcare, 2011).

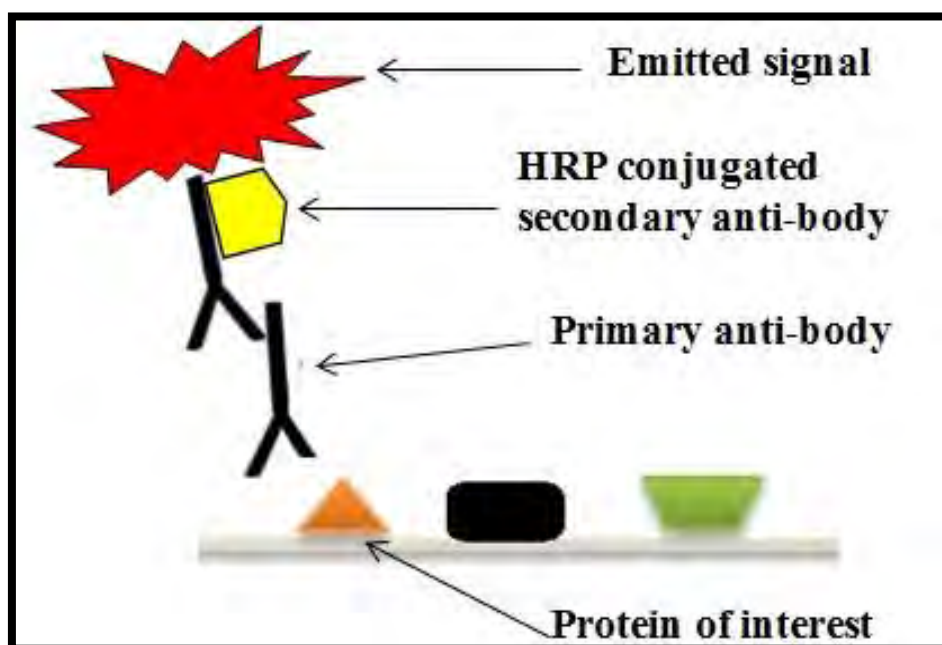


Figure 2.8: Signal emission as a result of anti-body antigen interactions.

2.5.1.5. Determining densitometry for analysis

Detection of signals using a camera based imager results in one or more visible protein bands on the membrane image. The molecular weight of the protein can be determined by comparing it to the molecular weight marker and the quantity of protein can be determined as this is related to band intensity (Healthcare, 2011).

To quantitate protein levels of the protein of interest normalisation to an internal reference such as housekeeping protein must be done. These housekeeping are constitutively expressed

proteins that maintain cell viability. The use of these proteins minimises protein loading errors and varying protein concentrations between each well from affecting overall results (Healthcare, 2011). The protein of interest is then quantified in relation to the housekeeping protein to determine relative band density.

2.5.2. Protocol

Western blot was run to determine protein expression of SIRT3, LON, Nrf2, PGC-1 α , p-CREB, NRF1 and HSP70. Crude protein of control, FA and NAM treated cells was isolated using CytobusterTM (Novagen, San Diego, CA, USA) supplemented with phosphatase and protease inhibitors (Roche, Mannheim, Germany, 05892791001 and 04906837001, respectively). Cytobuster (200 μ l) was added to flasks and incubated on ice for 10 minutes before being mechanically lysed. Cell lysates were decanted into 1.5 ml tubes and centrifuged (12,000 \times g, 10 minutes) to obtain crude protein. The BCA assay was used to quantify protein which was standardized to 1.5 mg/ml.

Samples were prepared in Laemmli buffer (dH₂O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, β -mercaptoethanol, 1% bromophenol blue) and electrophoresed (150V, 1 hour) in sodium-dodecyl-sulfate polyacrylamide gels (4% stacking, 10% resolving) using BioRad compact power supply. Protein was then transferred onto nitrocellulose membranes using the Trans-Blot[®] Turbo Transfer system (BioRad) (400mA, 45 minutes). Membranes were then blocked with 5% non-fat dry milk (NFDm) made up in Tris-buffer saline (TTBS) [0.5% Tween20, dH₂O, KCl, Tris, NaCl, pH 7.4] for one hour at RT. Membranes were then immune probed with primary antibody (1:1000 dilution in 5% NFDm) against Nrf2 (ab31163, Abcam), PGC-1 α (ab72230, Abcam), SIRT3 (ab86671, Abcam), phospho- CREB (9191, Cell Signaling Technology), HSP70 (4876, Cell Signaling Technology), NRF1 (12381, Cell Signaling Technology) and LONP1 (HPA002192, Sigma- Aldrich, St Louis, MO) for 1 hour at RT on a shaker then overnight at 4°C. Membranes were washed with TTBS (5 times, 10 minutes) and then incubated with secondary antibody conjugated to HRP [goat anti-mouse (31800); goat anti-rabbit (ab6112) 1:10 000 in 1% BSA] for 1 h at RT on shaker. Membranes were then washed with TTBS (5 times, 10 minutes). Protein bands were visualized using Clarity Western ECL Substrate (BioRad) detection reagent. Images were captured using gel documentation system Alliance 2.7 (UViTech, Cambridge, UK). UViTech Alliance Analysis software was used to analyse protein expression.

Membranes were stripped with 5% hydrogen peroxide, incubated in blocking solution (5% NFDm; 1 h; RT), rinsed thrice in TTBS and probed with HRP-conjugated antibody for the

house-keeping protein, β -actin (Sigma). The relative band intensity was normalised against β -Actin. Results were expressed as Relative band density (RBD).

2.6. Gene expression – Quantitative polymerase chain reaction

2.6.1. Introduction

Polymerase chain reaction (PCR) is a powerful and simple tool used to amplify a specific DNA sequence *in vitro* from a template strand. Two oligonucleotide primers complementary to the sites flanking the target region are chemically synthesised. These primers attach to each of the DNA template strand at the 3' ends. The enzyme DNA polymerase then incorporates deoxynucleotide triphosphates (dNTPs) to the 3' ends of the primers in a stepwise manner.

The PCR is performed in a thermocycler where it undergoes repetitive cycling of three incubation steps at different temperatures (Figure 2.9). The three steps include:

1. Denaturation: Double stranded (ds) DNA is denatured by heat (90°C) to form single stranded (ss) DNA.
2. Annealing: Complementary primers to the target sequence are annealed to the template DNA at a low temperature (55°C).
3. Extension: The annealed primers are extended by a DNA polymerase (Taq DNA polymerase, 72°C). The target copy is amplified upon each cycle. This results in exponential amplification of the original DNA fragment.

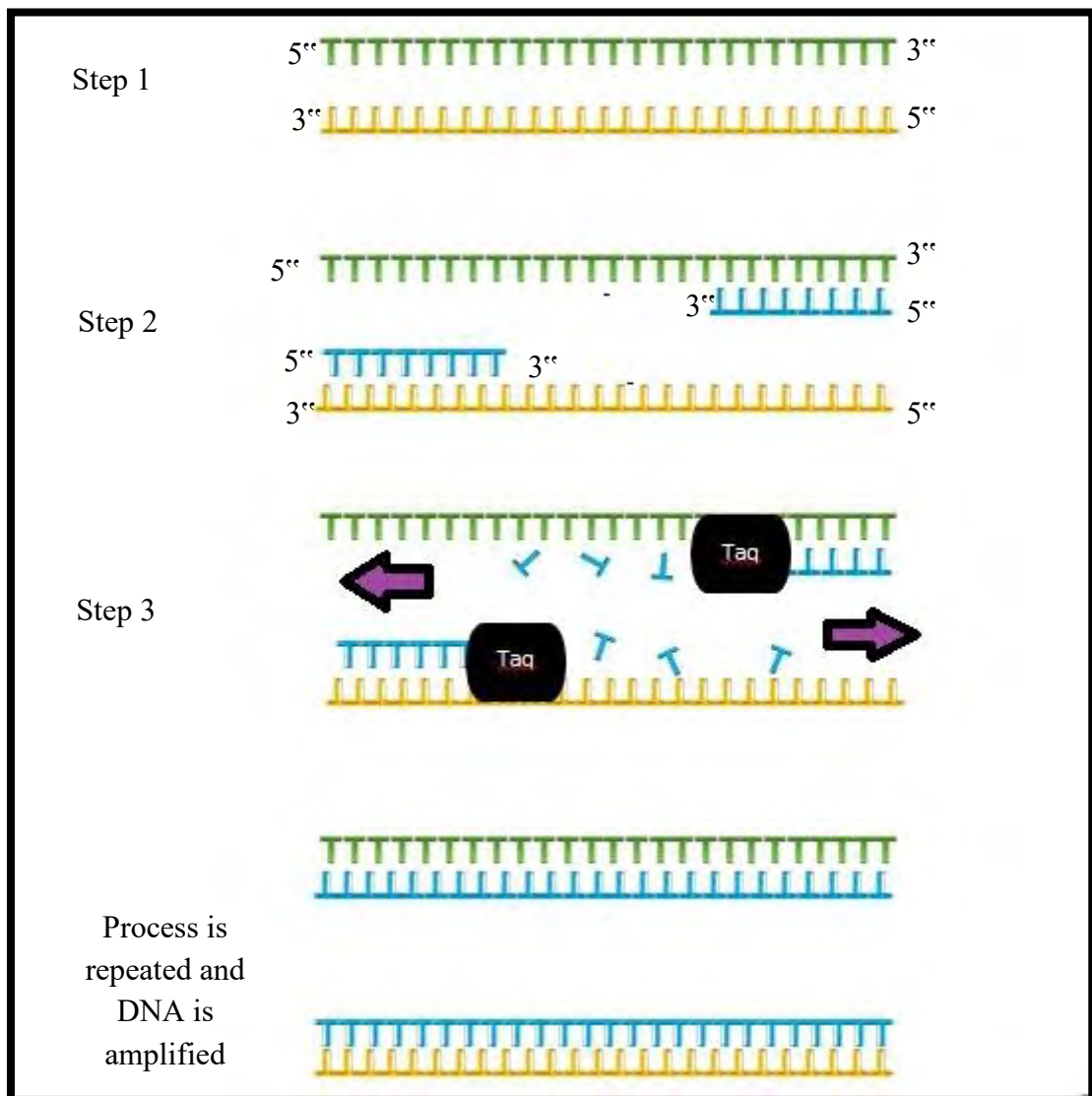


Figure 2.9: Steps of the PCR cycle leading up to DNA amplification (prepared by author).

Conventional PCR allows for the successful amplification of DNA, however accurate quantification is a limitation of this technique. Quantitative PCR, a variation of the original PCR process is used to determine the amount of product produced (i.e. the expression of target gene in a sample).

RNA is isolated from cells and reverse transcribed to ss complementary (c) DNA. This cDNA is used as the starting material for q-PCR. The q-PCR undergoes the same cycling steps as the conventional PCR. Quantification of DNA is made possible by adding a DNA-binding dye called SYBR Green to the reaction that can be detected after excitation. This dye binds to dsDNA amplicons and fluoresces in proportion to the ds DNA present (Figure 2.10). Along

with the gene of interest, samples are analysed for expression of a house keeping gene, and the amount of target DNA is reported relative to the amount of the house keeping gene for each sample.

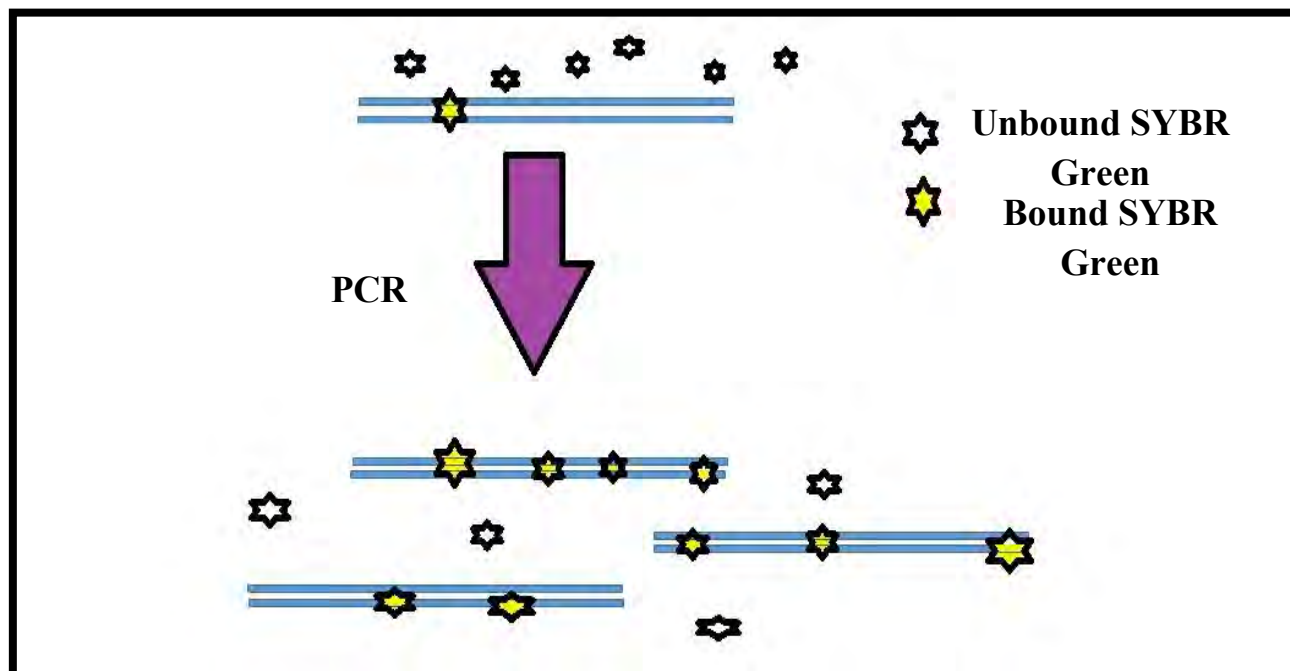


Figure 2.10: Fluorescence increases dramatically when dye molecules bind to dsDNA
(prepared by author)

2.6.2. Protocol

RNA was isolated from control, FA and NAM treated flasks with Qiazol reagent (Qiagen, Hilden, Germany). Briefly, 500 μ l Trizol was added to 500 μ l cells (2.5×10^6 in 0.1 M PBS) and incubated for 1h at -80°C . Chloroform (100 μ l) was then added and centrifuged (12,000Xg, 15 min, 4°C) followed by the addition of isopropanol (250 μ l, 1 h, -80°C) before centrifugation (12,000Xg, 20 min, 4°C). Samples were then washed with 500 μ l ethanol (75%) and centrifuged (7,400Xg, 15 min, 4°C). Following removal of ethanol, RNA pellets were re-suspended in 15 μ l of nuclease-free water, quantified (Nanodrop2000) and standardised (1,000 ng/ml). A 20 μ l reaction volume containing 1 μ l RNA template, 4 μ l 5X iScriptTM reaction mix, 1 μ l iScript reverse transcriptase and nuclease free water was used to synthesize cDNA (iScriptTM cDNA Synthesis kit, BioRad; catalog no 107-8890). Thermocycler conditions were 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and a final hold at 4°C .

Gene expression of *SIRT3* was assessed (Sense 5'-CGGCTCTACACGCAGAACATC-3'; Anti-sense 3-CAGCGGCTCCCCAAAGAACAC-5') using the iQ™ SYBR® Green PCR kit (Bio-Rad; 170-880) and carried out using CFX Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR was initiated with the following thermocycler profile: An initial denaturation for 8min at 95°C followed by 39 cycles of 95°C, denaturation for 15 sec, annealing for 1min at 50°C, and extension of 72°C for 30 sec. A final extension at 70°C was performed for 30 sec. Each measurement was done in triplicate and normalized against β -actin which was run under the same conditions and used as the housekeeping gene. Data was analysed using the method described by Livak and Schmittgen (2001) (Livak and Schmittgen, 2001) and represented as fold change relative to the housekeeping gene, *β -actin* (Sense 5'-TGACGGGTCACCCACACTGTGCCCAT-3'; Anti-sense 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'). Experiments were run in triplicate.

2.7. Assessment of caspase activity

2.7.1. Introduction

The Caspase Glo® 9 Assay and Caspase Glo® 3/7 Assay are homogenous luminescent assays that measure caspase 9 and caspase 3/7 activity respectively. Caspase 9 is an initiator caspase and plays a pivotal role in the intrinsic apoptotic pathway. Caspase 3/7 play key effector roles in the apoptotic programme and are responsible for the many biochemical characteristics associated with this process. These assays provide a luminogenic caspase 9 and 3/7 substrate in a buffer system that has been optimized for cell lysis, caspase and luciferase activity. The above mentioned caspases cleave the substrate and generate a luminescent signal as a result of the luciferase reaction (Figure 2.11). Caspase activity is proportional to the luminescent signal.

2.7.2 Protocol

The Caspase Glo® 9 Assay and Caspase Glo® 3/7 Assay kits (Promega, Madison, USA) were used to detect caspase activity. For both assays the same procedure was followed: FA treated and untreated cells were seeded into an opaque 96-well polystyrene plate in triplicate (20,000 cells/well in triplicate). The Caspase Glo® 9 and Caspase Glo® 3/7 reagents were prepared according to the manufacturer's instructions and 50µl of the reagent was added to the samples. The samples were then incubated in the dark at room temperature (30 mins). Following incubation period luminescence was detected and quantified using a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The data was expressed as mean relative light units (RLU).

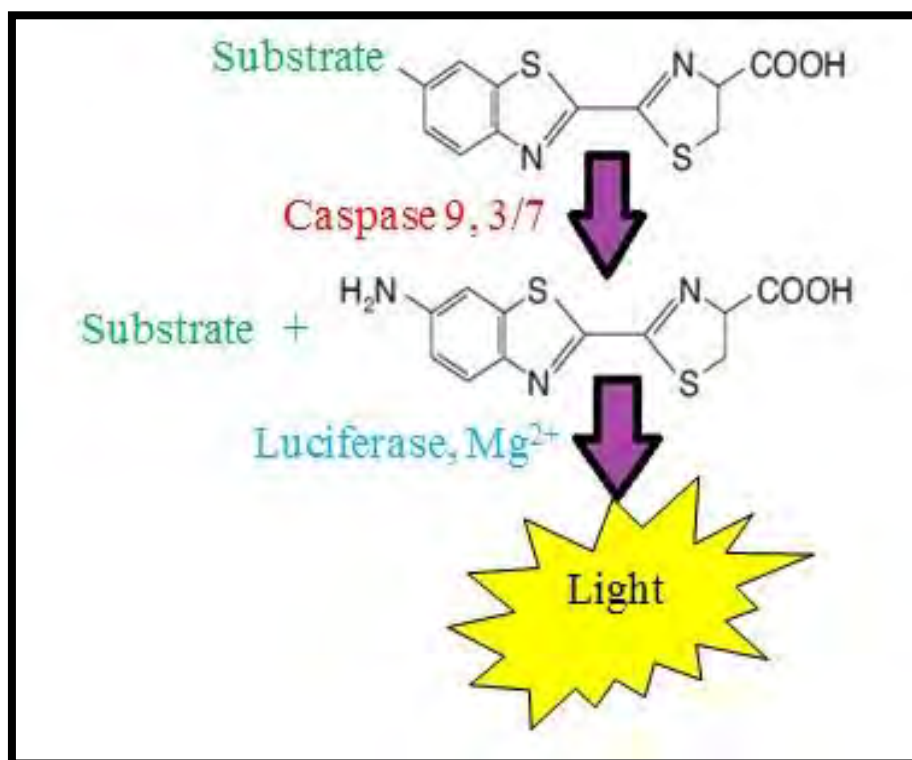


Figure 2.11: Schematic of the caspase activity assay (prepared by author).

2.8. Cytotoxicity – lactate dehydrogenase assay (LDH) assay

2.8.1. Introduction

Cell death can be evaluated by assays assessing cell membrane damage. Such assays are often based on the activity of cytoplasmic enzymes released by damaged cells. The amount of enzyme activity detected correlates to the extent of membrane damage. LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into culture supernatant upon loss of membrane integrity.

The LDH activity is measured by an enzymatic test. In the first step NAD^+ is reduced to $NADH/H^+$ by the conversion of lactate to pyruvate catalysed by LDH. In the second step diaphorase (catalyst) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which is reduced to formazan (Figure 2.12). The release of enzyme and its activity is proportional to cell membrane integrity.

2.8.2 Protocol

The LDH cytotoxicity detection kit (Roche, Mannheim, Germany) was used to measure cell death/membrane damage. To measure LDH activity, supernatant (100µl) was transferred into a 96-well microtitre plate in triplicate. Thereafter, substrate mixture (100µl) containing catalyst (diaphorase/NAD⁺) and dye solution (INT/sodium lactate) was added to the supernatant and allowed to react at RT for 25min. Optical density was measured spectrophotometrically at 500nm (Bio-Tek uQuant). Results are presented as mean optical density.

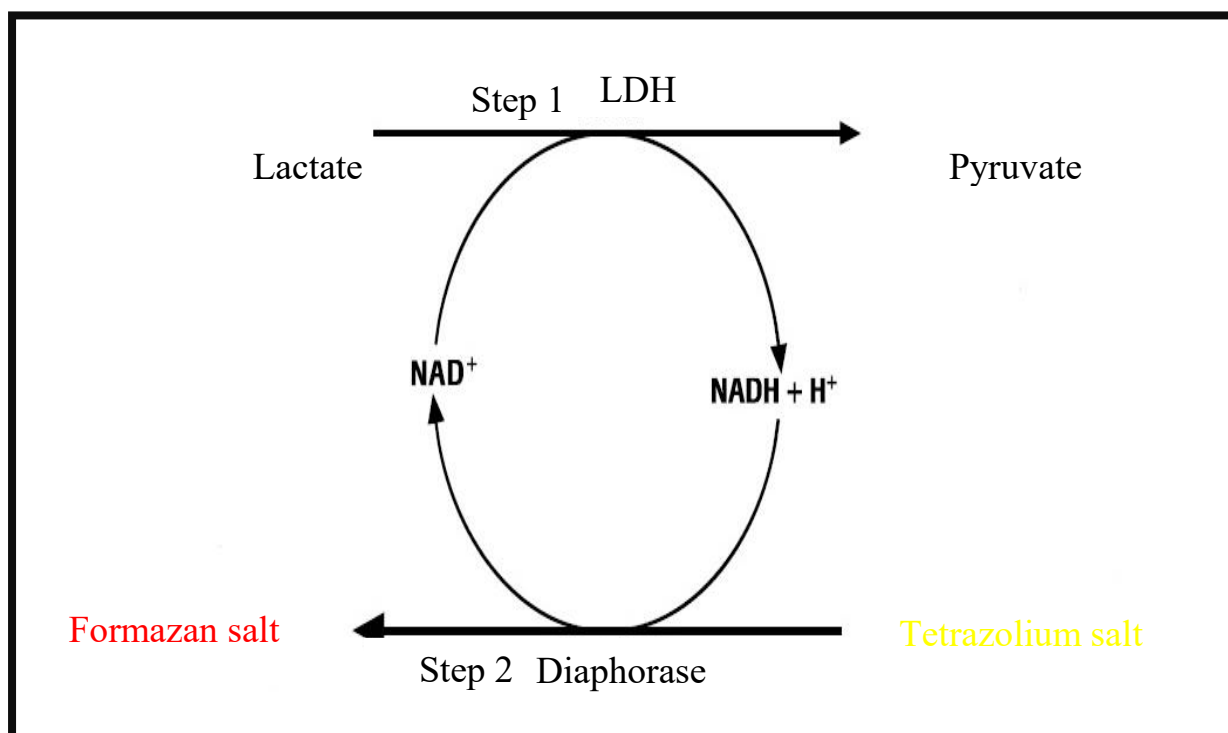


Figure 2.12: Reactions of the LDH cytotoxicity assay (prepared by author).

2.9 DNA damage – Single Cell Gel Electrophoresis (SCGE)

2.9.1 Introduction

The SCGE is a rapid and sensitive method for determining the extent of DNA damage in individual cells. For this assay cells are embedded in an agarose gel and lysed in a high salt detergent solution. This results in release of DNA that when subjected to electrophoresis under alkali conditions migrates toward the anode. A stain is used to visualise the extent of DNA strand breaks.

The images produced by this assay appear as “comets” with a head and a tail that can be measured. The head contains intact DNA while the tail contains migrating fragments of damaged DNA. These DNA fragments are smaller in size therefore migrate further through the gel than intact DNA (Figure 2.13).

2.9.2 Protocol

The SCGE assay was used to determine DNA fragmentation in the FA treated cells. Three slides per sample were prepared. A volume of 700µl of 2% low melting point agarose (LMPA) was pipetted toward the frosted end of a 76x26mm microscope slide. A 60x20mm coverslip was then placed over the molten agarose. It is paramount that the formation of air bubbles be avoided during this step. This first layer is needed to provide anchorage for subsequent layers of gel.

The cover slip was removed carefully using a needle point. A volume of 25µl cell suspension with approximately 20 000 cells from relevant treatments were transferred into a 1.5ml microcentrifuge tube along with 175µl of 1% LMPA at 37°C and 1µl GR red (staining solution). The resulting solution was stirred and immediately pipetted onto the first solid layer. The slides were then covered with cover slips and maintained at 4°C for ten mins. This second layer provides a meshwork in which the cells remain encapsulated and immobile.

The cover slip was once again removed and a third layer of gel was laid down. 200µl of 1% LMPA at 37°C was pipetted onto the second layer. Cover slips were placed on each of the slides and the final layer was allowed to solidify at 4°C for ten mins.

Once the gel had solidified the cover slips were removed and submerged in freshly prepared cold lysing solution. Slides were then incubated at 4°C for one hour and protected from light. The lysing solution (2.5M NaCl, 100mM EDTA, 1% Triton X-100, 10mM Tris (pH 10) and 10% DMSO) must be chilled so as to maintain the stability of the agarose gel layers. The lysis step is required to remove cell membranes, nucleoplasm and cytoplasm as well as dissolving nucleosomes since the solution is a hypertonic detergent.

After the elapsed incubation time the slides were removed from the lysing solution. Slides were then placed side-by-side into the electrophoresis tank with the frosted end closest to the cathode. The tank was filled with the prepared electrophoresis buffer (300mM NaOH, 1mM Na₂EDTA, pH 13) to a level approximately 20mm above the slides. The cells were then allowed to equilibrate for 20 minutes in the alkaline electrophoretic solution. This equilibration step is needed to unwind the DNA supercoils exposing alkali labile sites which appear as breaks.

Furthermore the high alkali concentration improves the resolving power of the assay without affecting sensitivity.

Following incubation the tank was sealed and a current of 300mA (25V) was applied 35min at RT using a Bio-Rad compact power supplier. This step is required to cause fragments of DNA to migrate toward the anode if there are breaks in the DNA. The DNA fragments are localized at the tail portion whilst the intact DNA is located at the head.

After electrophoresis the buffer was removed and the slides were washed three times with neutralization buffer (0.4M Tris, pH 7.4) for 5 min each to remove detergents and neutralize excess alkali. The slides were viewed using a fluorescent microscope (Olympus IXSI inverted microscope) using filter 4 (510-560 nm excitation and 590 nm emission filters). Images of 50 cells were taken per treatment, these were analysed Soft imaging system (Life Science – Olympus Soft Imaging Solutions v5) and expressed in μm .

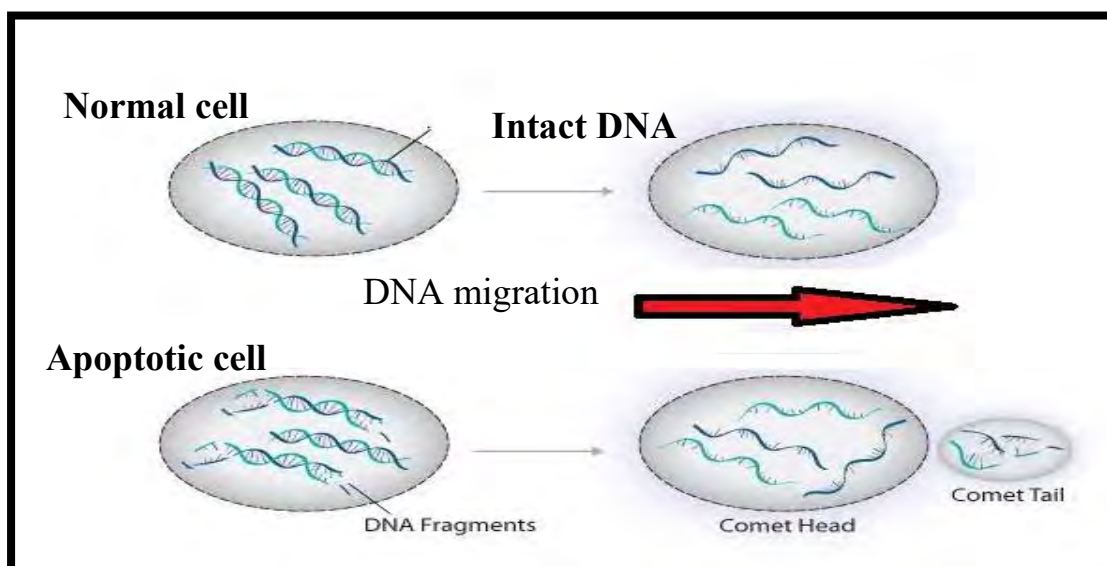


Figure 2.13: Principle of the SCGE assay (prepared by author)

2.10. Statistical analyses

Data was analysed using GraphPad prism V5.0 software (GraphPad Software Inc., La Jolla, USA.) Data was considered to be statistically significant with a p value < 0.05 . Unpaired t-test with Welch correction (data reported as mean \pm standard deviation) or the one-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple group comparison (data is presented as 95% CI) was used to determine statistical significance.

The unpaired t-test is employed to compare two groups of data (control vs. treatment) when observations are not equal and the data is continuous yet randomly distributed (Barile, 2013). Welch's correction assumes that both data sets are sampled from Gaussian populations but does not assume that these data sets have an equal standard deviation (Prism, 2016).

The ANOVA test compares the total variation present in more than two groups of data sets (Barile, 2013). The Bonferonni test compares every pair of means but are selected based on experimental design. When comparing multiple pairs of grouped data at once the individual p value can not be determined in the normal way, instead a significance level is set and comparisons are statistically significant based on this threshold.

CHAPTER 3: RESULTS

3.1. Mitochondrial Output

To determine the effect of FA on mitochondrial output, cell viability and intracellular ATP levels were assessed.

3.1.1. Cell viability

A dose response curve was obtained using serially diluted concentrations of FA (0-500 μ g/ml) in HepG2 cells over 24h. The curve showed that FA decreased metabolic activity in a dose dependent manner; 104 μ g/ml FA caused a 50% inhibition (IC_{50}) of metabolic activity and this concentration was used in all subsequent assays (Figure 3.1).

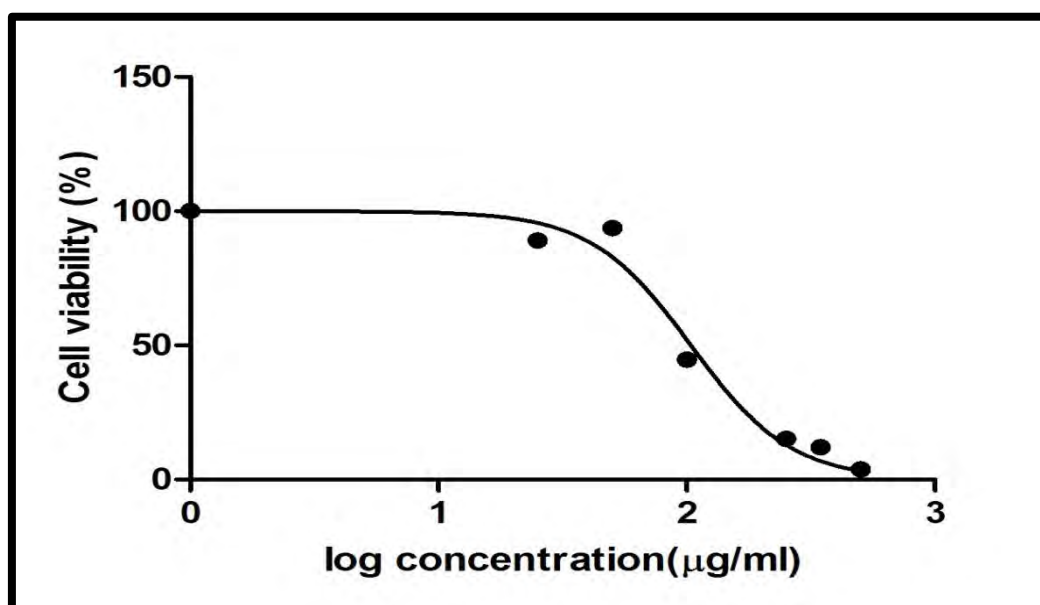


Figure 3.1: A dose dependent decline in metabolic activity following treatment with varying concentrations (0, 25, 50, 100, 250, 350, 500 μ g/ml) of FA.

3.1.2. Intracellular ATP levels

Intracellular ATP level was measured using a luminometric assay. Figure 3.2 shows that FA caused a significant ($p = 0.0062$) decrease in ATP levels ($0.815 \pm 0.0682 \times 10^6$ RLU) when compared to the control ($2.044 \pm 0.1582 \times 10^6$ RLU).

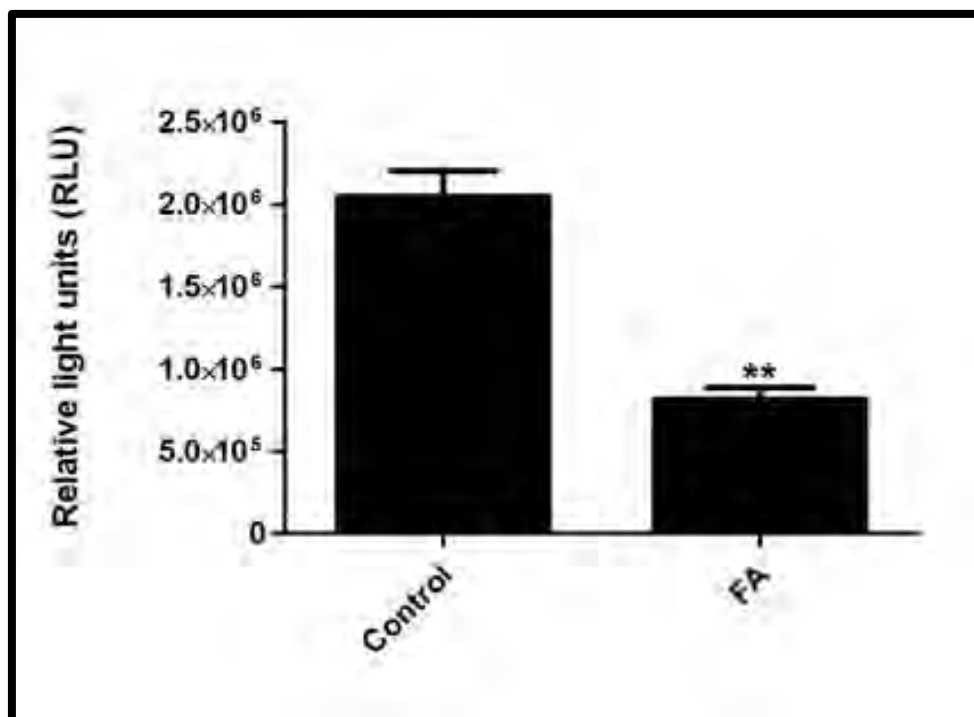


Figure 3.2: Intracellular ATP levels were significantly depleted (** $p=0.0062$) in FA treated cells. Results are presented as mean \pm standard deviation.

3.2. Oxidative stress and detoxification

3.2.1. Oxidative stress

Lipid peroxidation was used as a measure of oxidative stress. MDA levels were significantly increased in FA treated cells when compared to control cells ($P = 0.0002$; 95% CI, -14.02×10^3 to -6.644×10^3). Higher MDA levels were also observed in FA treated cells relative to NAM treated cells ($p = 0.0002$; 95% CI: 5.978×10^3 to 13.36×10^3). NAM treatment did not exhibit significant changes to ROS levels when compared to control cells (Figure 3.3).

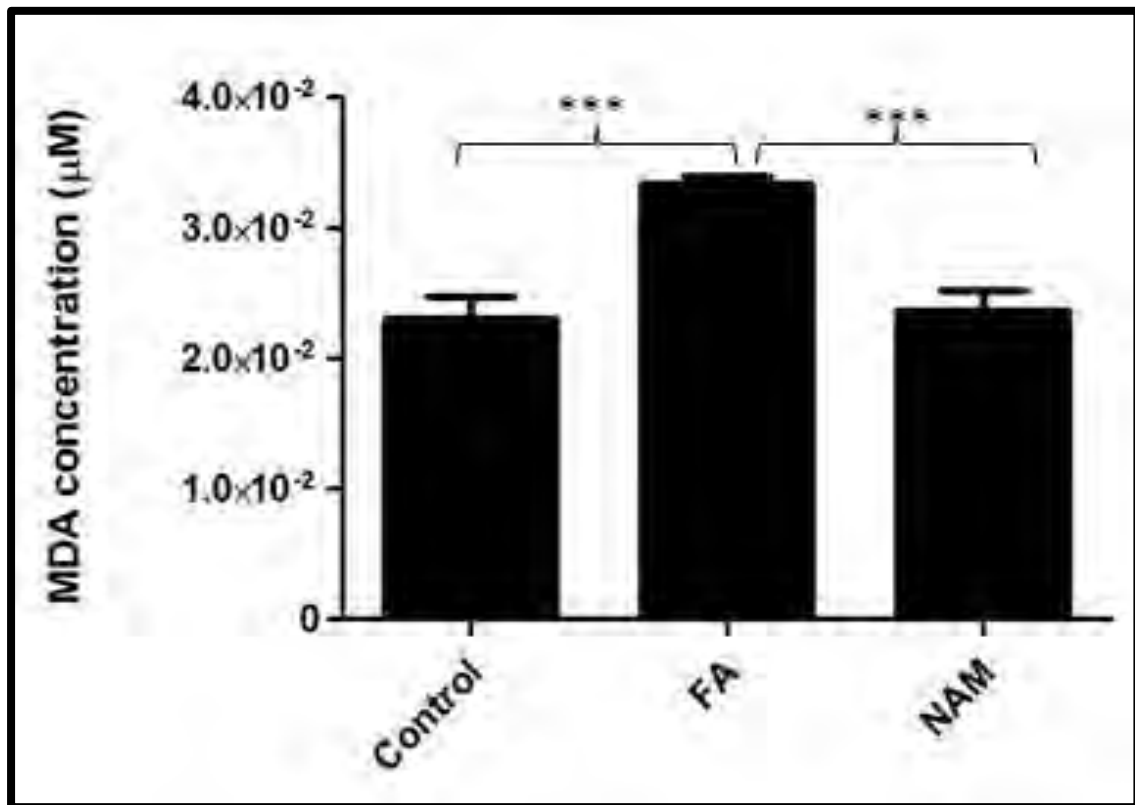


Figure 3.3: FA induced oxidative stress as indicated by elevated MDA levels (***) $p = 0.0002$ when compared to NAM and the untreated control. Results are expressed as mean \pm standard deviation.

3.2.2. Phase 2 detoxification

Increased oxidative stress by FA may interfere with cellular anti-oxidant systems such as Nrf2 expression. FA significantly increased Nrf2 expression (western blots) when compared to both untreated controls ($p < 0.0014$; untreated vs. FA, 95% CI, 3.058×10^{-2} RBD to -0.367×10^{-2} RBD; FA vs. NAM, 95% CI 1.493×10^{-2} RBD to 3.9×10^{-2} RBD) but no significant changes were observed between untreated cells and NAM treated cells (Figure 3.4).

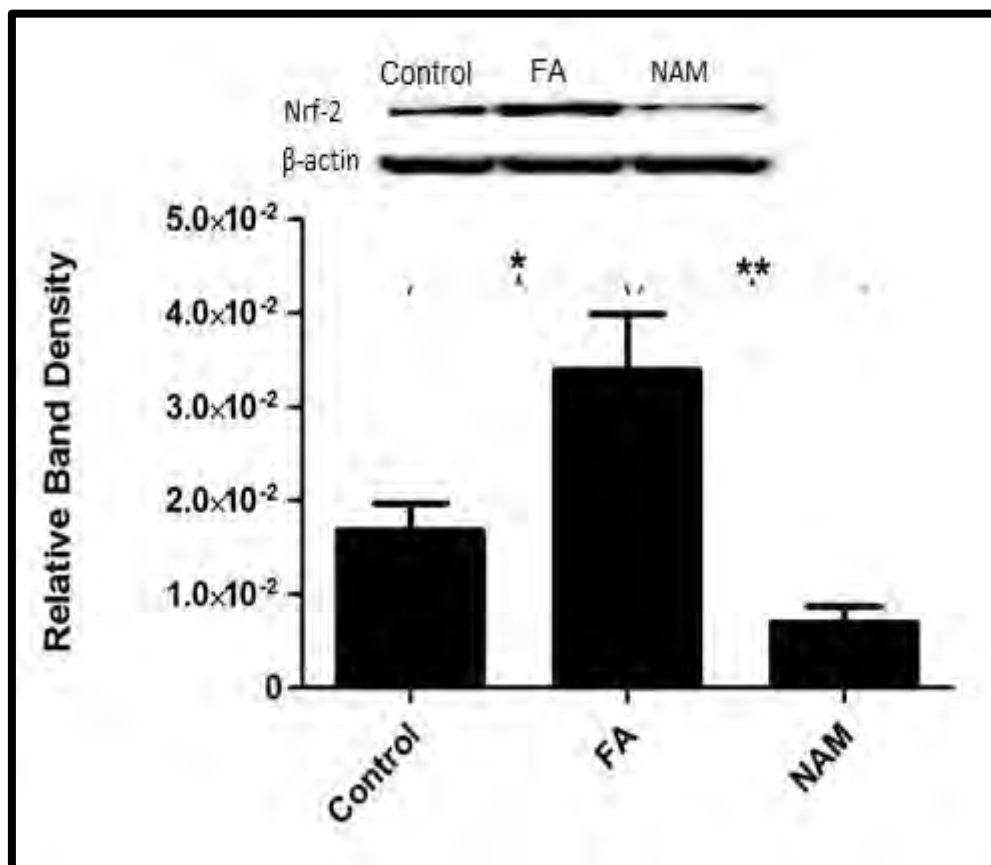


Figure 3.4: The regulator phase II detoxification responses (nrf-2) was found to be significantly elevated (**/***p < 0.0014) in FA treated cells when compared to NAM and the untreated cells.

3.3. Mitochondrial stress

3.3.1. Sirtuin 3

Sirtuin 3 is a key regulator of mitochondrial function and stress responses. Changes to protein and gene expression of SIRT3 in FA and NAM (positive control) treated cells relative to untreated cells were examined. Since NAM is a known inhibitor of SIRT3 activity, the activity of SIRT3 in response to FA can be better understood.

Following exposure to FA, a significant decrease in SIRT3 protein expression in comparison to both sets of control cells (p = 0.0012; untreated vs. FA, 95% CI, 0.2884×10^{-3} RBD to 0.7275×10^{-3} RBD; FA vs. NAM, 95% CI -0.5893×10^{-3} RBD to -0.1502×10^{-3} RBD) was observed (Figure 3.5A).

To validate the reduced expression of SIRT3 at the protein level *SIRT3* gene expression was investigated with qPCR. These results show gene expression to be significantly down-regulated in response to FA but not NAM when compared to untreated cells (Figure 3.5B) (p = 0.0007; untreated vs. FA, 95% CI, 0.7160 to 1.473; FA vs. NAM, 95% CI -1.164 to -0.4069).

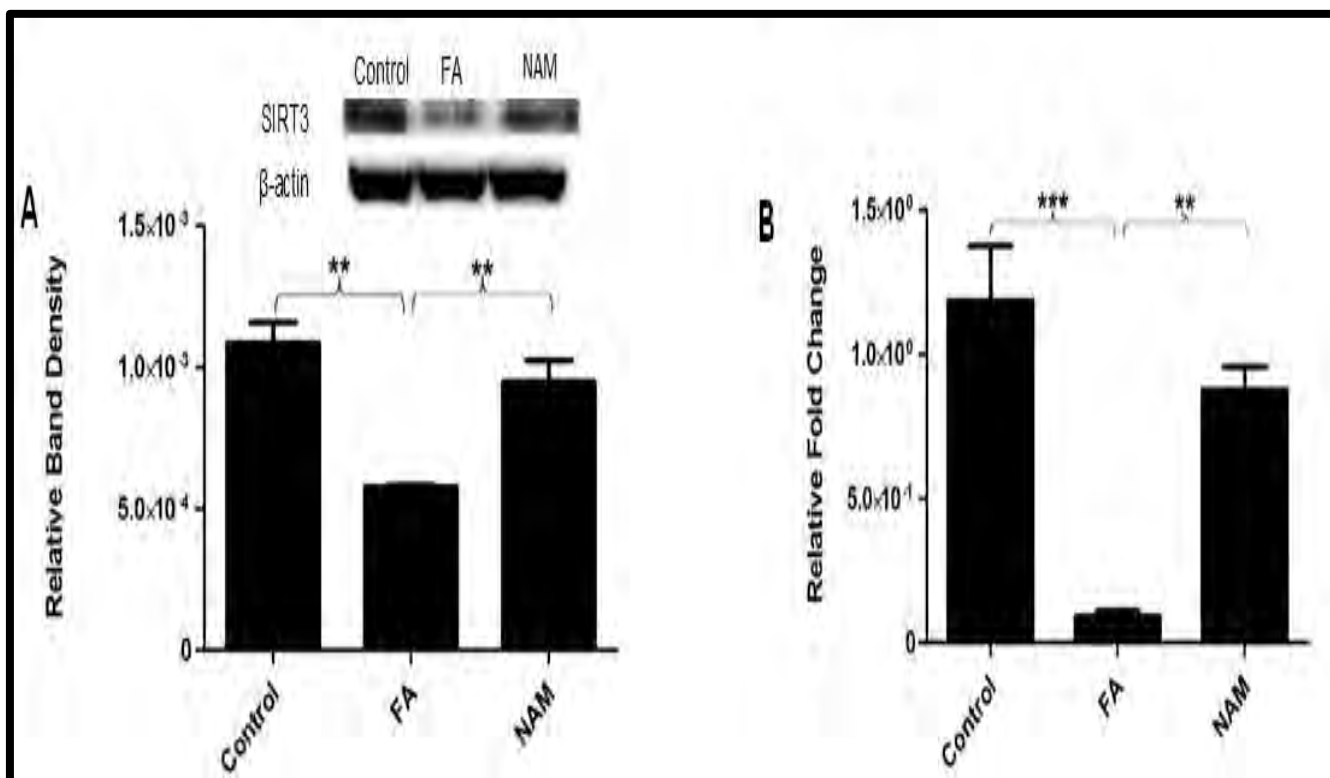


Figure 3.5: FA significantly decreased both SIRT3 protein (A, **: $p = 0.0012$) and mRNA (B, ***/** $p = 0.0007$) expression levels

3.3.2. Lon Protease

The down-regulation of SIRT3 has implications for mitochondrial stress responses. Protein expression of LON, a key mitochondrial stress response protein was measured. FA significantly decreased expression of LON in the untreated control ($p = 0.0044$; untreated vs. FA, 95% CI, 0.1997 RBD to 1.287 RBD; untreated vs. NAM, 95% CI 0.4179 RBD to 1.411 RBD). The difference in LON expression induced by FA as compared to NAM was not significant (Figure 3.6).

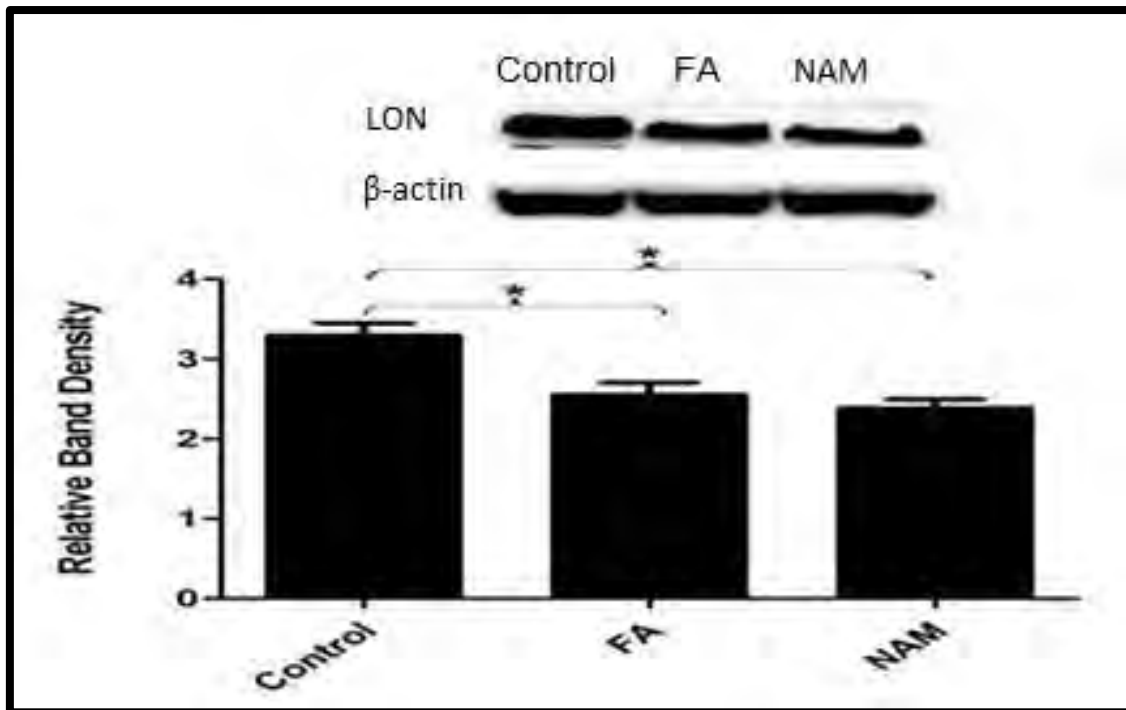


Figure 3.6: LON protein expression was significantly down-regulated (**: $p = 0.0044$) in cells treated with FA and NAM.

3.4. Mitochondrial biogenesis

Since both SIRT3 and LON are involved in mitochondrial biogenesis, the effects of FA in this process were further investigated.

3.4.1. Peroxisome proliferator-activated receptor γ co-activator α (PGC-1 α)

The effect of FA on PGC-1 α protein expression, the master regulator of mitochondrial biogenesis (Ventura-Clapier et al., 2008) was determined and is shown in figure 3.7. Significantly lower levels of PGC1- α protein expression was observed in FA and NAM treated cells when compared to the untreated control ($p = 0.0005$; untreated vs. FA, 95% CI, 0.09168 RBD to 0.2127 RBD; untreated vs. NAM, 95% CI 0.09206 RBD to 0.2274 RBD). However no statistical significance was found between NAM and FA treated cells.

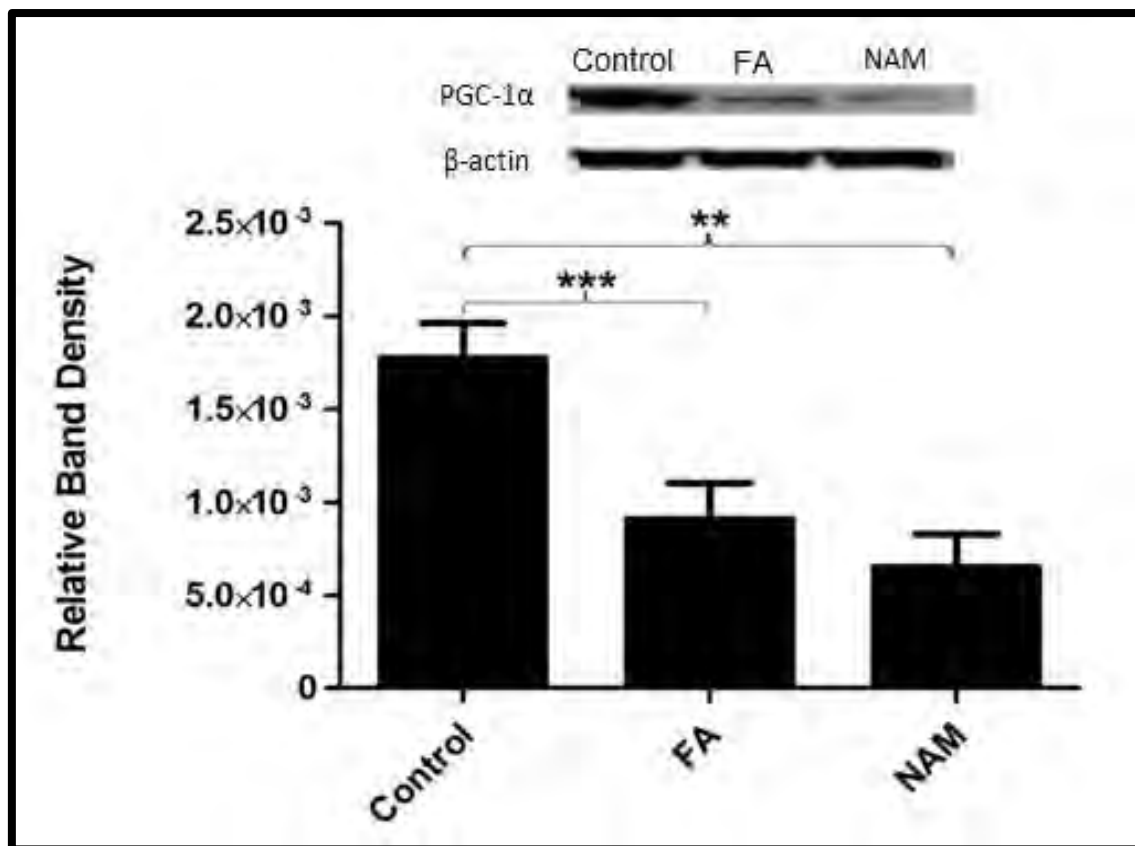


Figure 3.7: FA significantly decreased the expression of PGC-1α (***: $p = 0.0005$).

3.4.2. Phosphorylated cAMP response element binding protein (p-CREB).

The expression of the mitochondrial biogenesis regulatory protein p-CREB was examined and found to be significantly decreased in response to FA and NAM stimulation ($p = 0.0008$; untreated vs. FA, 95% CI, 0.361×10^{-3} RBD to 1.362×10^{-3} RBD; untreated vs. NAM, 95% CI 0.6213×10^{-3} RBD to 1.622×10^{-3} RBD, Figure 3.8).

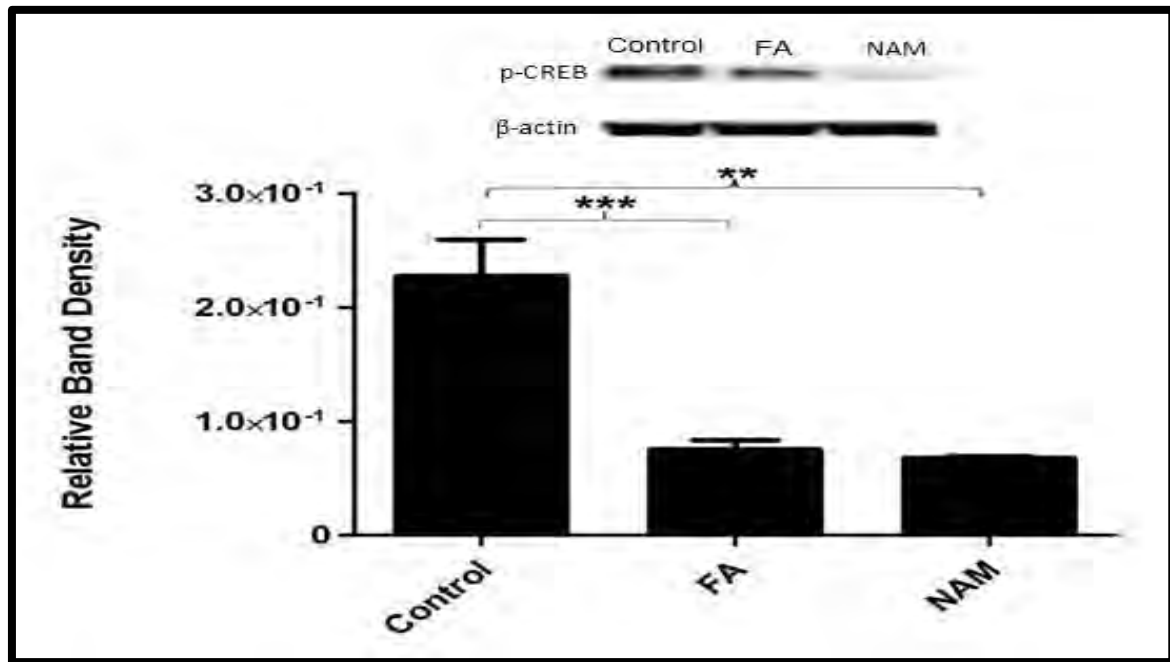


Figure 3.8: The expression of p-CREB was significantly down-regulated in response to both FA and NAM treatment (***/**: $p = 0.0008$)

3.4.3. Nuclear respiratory factor 1 (NRF-1)

Mitochondrial biogenesis is regulated at the transcriptional level by NRF1. As seen in figure 3.9 both FA and NAM induced significant decreases in NRF1 expression in comparison to untreated cells ($p = 0.0004$; untreated vs. FA, 95% CI, 0.1124 RBD to 0.2121 RBD; untreated vs. NAM, 95% CI 0.1177 RBD to 0.2270 RBD) but no significant changes were noted between FA and NAM treated cells.

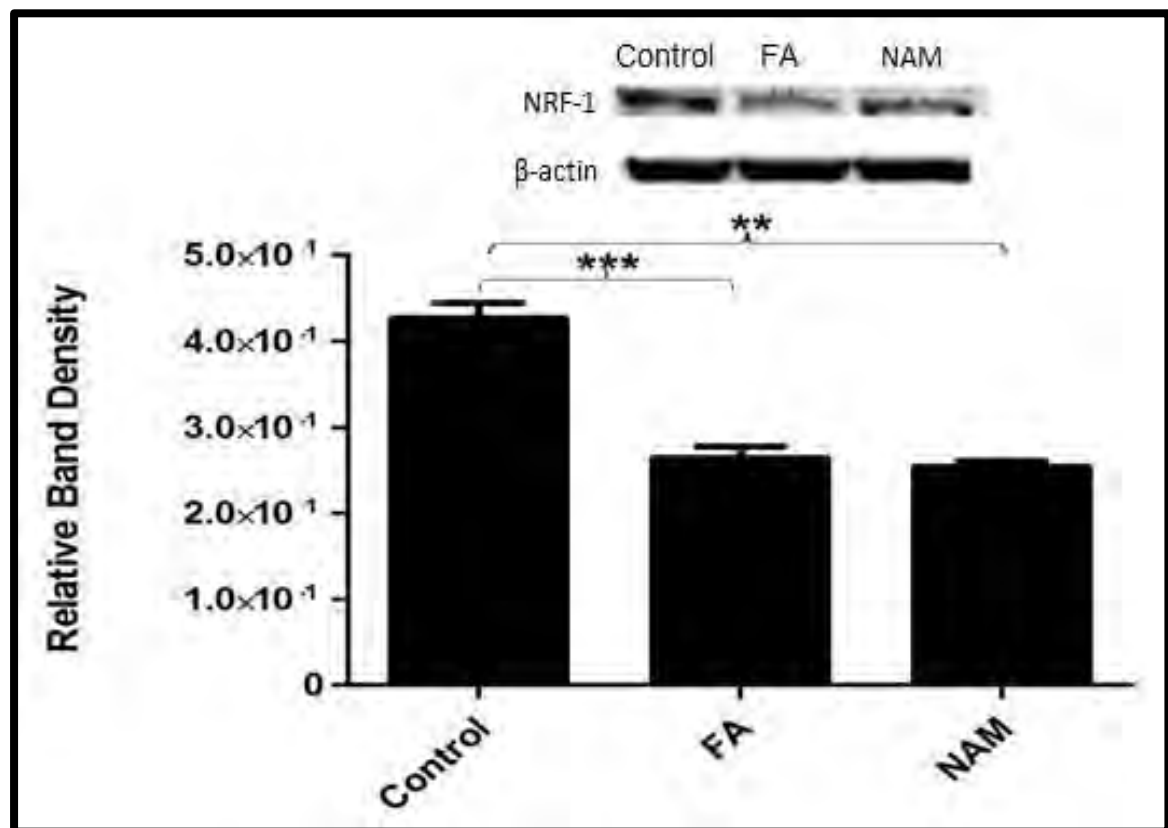


Figure 3.9: Both the FA and NAM stimulated cells exhibited significantly decreased protein expression of NRF-1 when compared to untreated cells (***: $p = 0.0004$)

3.4.4. Heat shock protein 70 (HSP70)

The chaperone protein HSP70 expression was determined. FA was seen to significantly decrease protein expression of HSP70 when compared to untreated and NAM stimulated cells ($P = 0.0102$; untreated vs. FA, 95% CI, 0.5672 RBD to 3.234 RBD; FA vs. NAM, 95% CI 3.142 RBD to -0.4758 RBD, Figure 3.10)

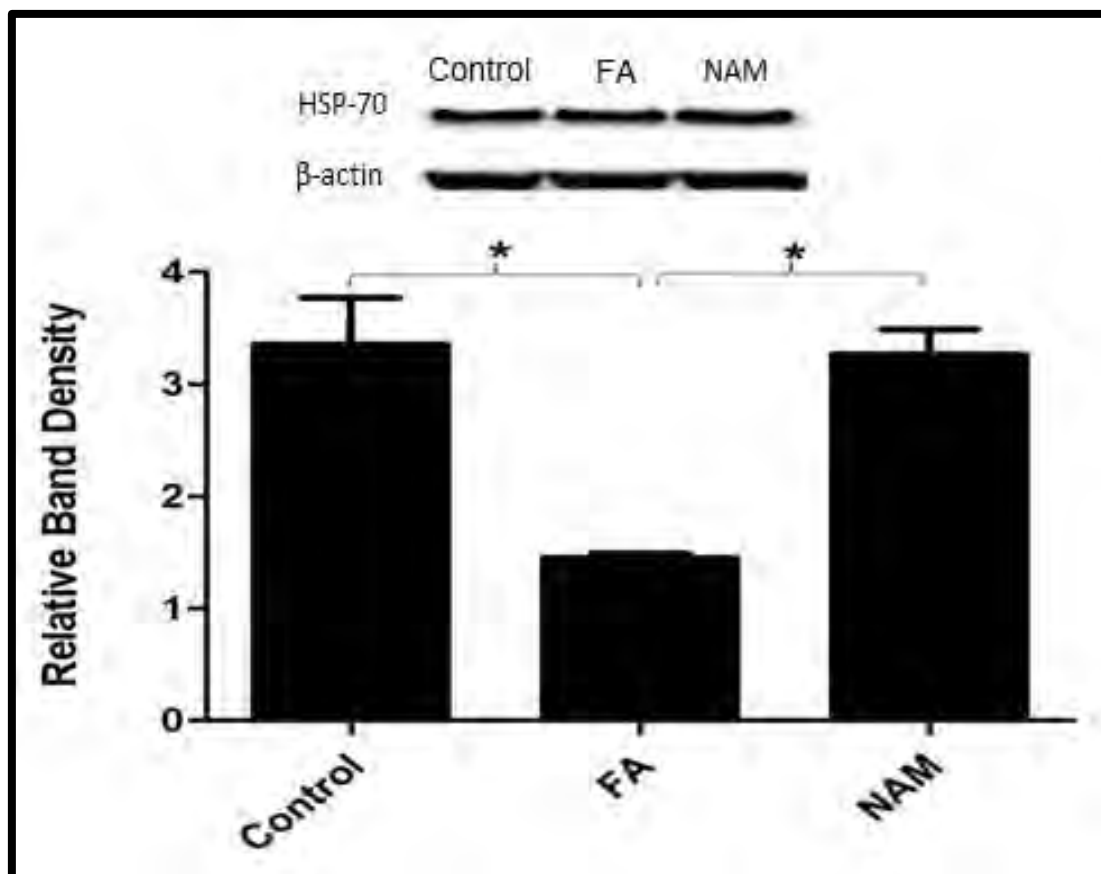


Figure 3.10: The expression of HSP70 was significantly decreased in response to FA stimulation when compared to both NAM and the untreated control cells (* $p = 0.0102$).

3.5. Cell damage and death

Since both mitochondrial and oxidative stresses culminate in cell damage/death the effect of FA on cell membrane integrity/cytotoxicity and caspase activity were investigated.

3.5.1. Membrane integrity and cytotoxicity

Seeing that MDA (by-product of lipid peroxidation) levels were increased we investigated the effect of FA on cell membrane integrity. FA induced significant cell membrane damage when compared to control cells as measured by increased LDH activity in FA treated cells ($p < 0.0001$, 2.902 ± 0.1385 vs. 0.8328 ± 0.06286) as shown in figure 3.11. The elevated LDH level is also an indicator of cytotoxicity/necrosis.

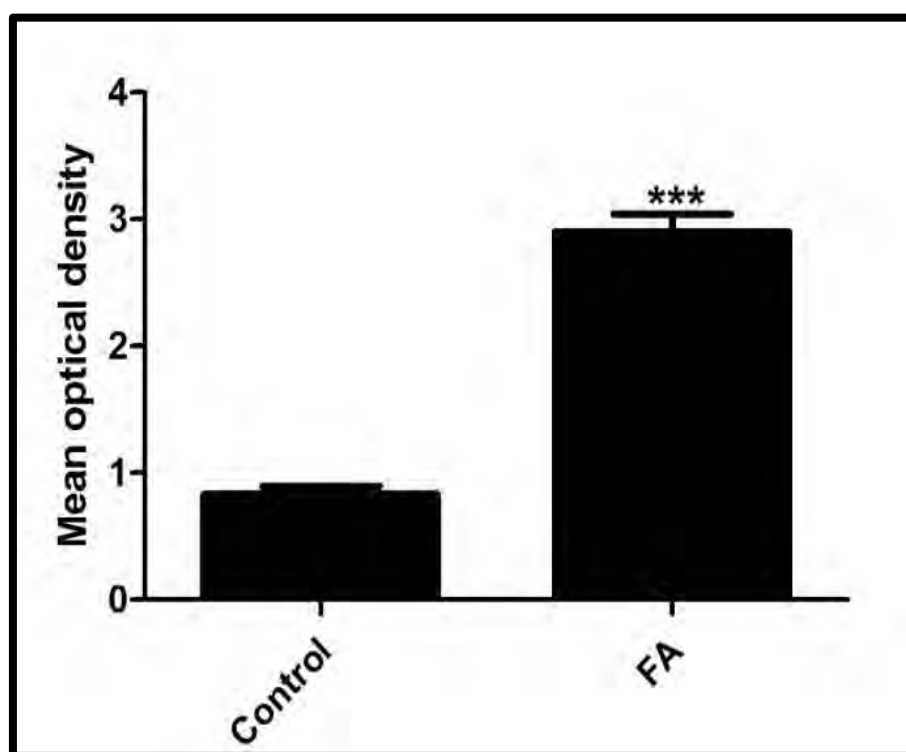


Figure 3.11: FA caused significant membrane damage and is cytotoxic to the HepG2 cell line as indicated by the LDH assay (**p<0.0001).

3.5.2. Caspase activity

The activities of caspase 9, the intrinsic apoptotic initiator and the executioner caspases 3 and 7 were measured. These results, presented in table 3, show FA significantly increased ($p= 0.0032$) the activity of caspases 3 and 7 when compared to control cells (7.482 ± 0.4973 vs. 24.040 ± 1.551). However no significant difference was observed in caspase 9 activity.

Table 2 Caspase activity in FA treated cells.

<u>Caspase</u>	<u>Mean \pm SD (RLU X 10^3)</u>		<u>p-value</u>
	Control	FA	
9	175.6 ± 9.423	166.30 ± 14.710	0.3340
3/7	7.482 ± 0.4973	24.040 ± 1.551	0.0032**

Results are presented as mean \pm Standard deviation (SD) and in relative light units (RLU)

CHAPTER 4: DISCUSSION

FA is produced by *Fusarium spp*, a ubiquitous soil fungus, and contaminates many agricultural products (Bacon et al., 1996), to date the belief was that it possessed low to mild toxicity. The liver is susceptible to toxic insult (Fenton, 2002, Wang and Ng, 1999). Hepatocytes are densely populated with mitochondria which serve as integrators for several metabolic pathways and regulators of hepatocyte survival. Hepatocyte damage is strongly associated with mitochondrial dysfunction coupled with increased ROS production (Esposti et al., 2012).

Previous studies have implicated ROS generation as a major contributor to FA induced toxicity (Hirai et al., 2005, Iwahashi et al., 1999, Jiao et al., 2014) whilst other studies showed FA chelation as a toxic mechanism (Ruda et al., 2006, Stack et al., 2004). The molecular mechanisms of FA toxicity, however, are not fully elucidated. SIRT3 regulates many aspects of mitochondrial function including ATP generation and stress responses (Chen et al., 2014). SIRT3 ideally functions as a mitochondrial fidelity protein and a loss of its function can result in cellular damage and eventual death.

The results show that FA decreased cell viability in a dose dependent manner, with a drastic decrease at higher concentrations (Figure 3.1). The MTT assay measures cell viability based on the generation of reducing equivalents by metabolically active cells (Nikzad et al., 2014). Telles-Pupulin et al. (1998) showed that FA impedes oxidative reactions of the Krebs's cycle by inhibiting α -ketoglutarate dehydrogenase and succinate dehydrogenase (Telles-Pupulin et al., 1998); α -ketoglutarate dehydrogenase regulates metabolic flux via the Krebs's cycle and catalyzes the conversion of α -ketoglutarate to succinyl-CoA producing NADH and directly provides electrons for the respiratory chain. Succinate dehydrogenase oxidizes succinate to fumarate producing FADH₂ and transfers electrons from succinate to ubiquinone thus playing a role in the Krebs's cycle and ETC (Champe et al., 2005). These results seem to be in agreement with studies that show FA interferes with reducing equivalent metabolism.

SIRT3 down-regulation at both the protein and gene levels (Figure 3.5A and 3.5B) shows that FA may selectively target the mitochondrion. Also, FA is a weak acid and this may also explain its mitochondrial toxicity (weak acids disrupt the hydrogen gradient across the mitochondrial membrane and decreases ATP production). The decreased SIRT3 protein expression can be attributed to its decreased gene transcript levels. It is also possible that FA, an efficient chelator of zinc (Stack et al., 2004), directly down regulated SIRT3 protein expression. SIRT3 contains a conserved enzymatic core with two domains, including a large Rossmann fold domain that binds NAD⁺ and a small domain formed by two insertions of the large domain binding to a zinc

atom (Nogueiras et al., 2012). FA may have caused loss of functional stability of SIRT3 through removal of the zinc ion.

FA severely depleted intracellular ATP levels (Figure 3.2). Ahn et al (2008) showed decreased respiration and ATP levels in SIRT3 double knock-out mice livers (Ahn et al., 2008), and is in agreement with the human *in vitro* model data in Figure 3.2. SIRT3 targets NADH dehydrogenase and succinate dehydrogenase and stimulates their activity (Finley et al., 2011, Chen et al., 2014). FA is structurally similar to NAM and may inhibit SIRT3 activity through competitive binding at the NAD⁺ site. Furthermore, these complexes contain iron-sulphur clusters (Champe et al., 2005, Atamna et al., 2002); FA can chelate iron *in vitro* (Hirai et al., 2005), and this would prevent the transfer of electrons in the ETC.

Furthermore, it was shown that FA directly suppressed the ETC by inhibiting ATP synthase activity (Telles-Pupulin et al., 1998). Thus FA may directly or indirectly inhibit mitochondrial enzymes as well as reducing equivalent metabolism and decrease ATP synthesis.

FA significantly elevated levels of MDA, a byproduct of lipid peroxidation and marker of oxidative stress (Figure 3.3). This is in agreement with Jiao et al (2014) (Jiao et al, 2014). Although ROS is damaging to a variety of cellular macro-molecules, membrane lipids are especially sensitive to free radicals due to the presence of PUFAs (Tretter and Adam-Vizi, 2005). The inhibition of the ETC by FA is a likely cause of the highly oxidative environment. It was shown that inhibition of the ETC, particularly at complex I and II leads to the increased generation of ROS (Chen et al., 2007). FA itself is thought to induce oxidative stress through chelation of iron and enhancement of the Fenton reaction (Hirai et al., 2005, Iwahashi et al., 1999). The nitrogen atom in the pyridine ring and the oxygen atom in the carboxyl group, may act as a chelator and enhance the Fenton reaction leading to formation of the hydroxyl radical (Iwahashi et al., 1999).

Induction of the phase 2 response is an important cellular defense to oxidative stress and is dependent on the release of Nrf2 from its repressor KEAP-1, a zinc thiol protein. Zinc is bound to reactive cysteine thiols of Keap1 and is displaced by electrophiles. Release of zinc alters the conformation of Keap1 thereby allowing translocation of Nrf2 to the nucleus (Dinkova-Kostova et al., 2005). FA significantly increased in Nrf2 expression (Figure 3.4). However, given that KEAP-1 is a zinc thiol protein it may be possible that FA chelates this ion and enhances the release of Nrf2. Despite elevated Nrf2 levels, the increased oxidative stress in the HepG2 cells still persisted. This may be due to the chelation of zinc, a critical ion involved in the activation and transcriptional function of Nrf2 (Ngo et al., 2013).

SIRT3 acts to dampen oxidative stress through enhanced expression and activity of SOD2 (Tao et al., 2010, Qiu et al., 2010). SIRT3 expression is normally up-regulated by oxidative stress (Flick and Lüscher, 2012); however our results show that FA decreased its expression at the gene level (Figure 3.5B). Kong et al. (2010) showed that PGC-1 α stimulated SIRT3 expression at the gene and protein levels (Kong et al., 2010) via CREB phosphorylation (Flick and Lüscher, 2012) and SIRT3 in turn up-regulated PGC-1 α expression –these proteins are regulated by a positive feedback loop (Chen et al., 2014). The results support this as FA is shown to disrupt this feedback loop by down regulating SIRT3, PGC-1 α and p-CREB (Figure 3.5A, 3.5B, 3.7, 3.8). Although no direct regulation of PGC-1 α by SIRT3 has been reported, a study showed that SIRT3 expression is required for the induction of oxidative responses by PGC-1 α (Kong et al., 2010). Interestingly inhibition of SIRT3 by NAM resulted in similar reductions to PGC-1 α and p-CREB protein expression when compared to FA treated cells, thus supporting the hypothesis that FA can dysregulate SIRT3 activity through competitive inhibition.

Despite FA and NAM inhibiting SIRT3 and resulting in similar reductions to PGC-1 α protein expression, no significant elevation in MDA levels was observed in NAM treated cells as compared to untreated controls. There was also no difference in the expression of Nrf2 by NAM. These results are supported by a study in SIRT3 knock-out mouse embryonic fibroblasts that showed oxidative stress to only be significantly elevated upon induction of cellular stress (Kim et al., 2010).

The removal of oxidatively damaged proteins via proteolytic degradation is an important cell defense to high oxidative stress. Oxidized mitochondrial proteins must be removed rapidly to prevent their aggregation, cross linking and toxicity. LON, an ATP dependent protease, catalyzes the degradation of oxidatively damaged proteins in the mitochondrial matrix (Bota et al., 2005). LON expression was decreased after treatment with both FA and NAM (Figure 3.6). Given that Nrf2 expression was up-regulated and SIRT3 expression down regulated by FA, it is likely that FA induced LON disruption at the post-translational level. This is supported by inhibition of SIRT3 with NAM showing similar reductions in LON expression. Furthermore the depletion of ATP by FA will prevent catalytic activity of LON inhibiting proteolytic degradation of oxidized proteins. FA induced mitochondrial stress in HepG2 cells is further aggravated by loss of LON function.

Since FA induced both oxidative and mitochondrial stress, we then determined its effect on mitochondrial biogenesis. Increased mitochondrial biogenesis aids cells to prevent mitochondrial lesions and maintain mitochondrial integrity (Esposti et al., 2012). PGC-1 α is known to enhance NRF1 dependent gene expression by acting as a co-activator. NRF1 (a

transcription factor) activates the expression of oxidative phosphorylation components and the expression of Tfam resulting in increased expression of nuclear mitochondrial genes and mitochondrial DNA replication (Piantadosi and Suliman, 2006). A decrease in NRF1 expression by FA was noted (Figure 3.9). Kong et al (2010) found that inhibition of SIRT3 expression diminished the PGC-1 α induction of mitochondrial biogenesis. They proposed that NRF1 is a substrate of SIRT3 however further studies are required to determine the molecular mechanism by which SIRT3 controls mitochondrial biogenesis (Kong et al., 2010).

Most genes encoding mitochondrial proteins are located in the nucleus rather than in the mitochondrial genome, these proteins must be imported into the mitochondria. This involves complex folding and assembly processes to ensure proper enzyme activity (Voos, 2013).

HSP70 is involved in mitochondrial protein homeostasis and by mediating translocation and folding reactions (Voos, 2013). The results show decreased expression of HSP70 by FA (Figure 3.10). HSP70 co-ordinates two calcium ions one of which contributes to protein stability while the other is needed to carry out ATP hydrolysis (Sriram et al., 1997), thus FA may affect both stability and activity of HSP70 through chelation of calcium. Additionally FA mediated depletion of ATP will impede folding reactions of HSP70 since ATP hydrolysis is coupled with its molecular chaperone function (Voos, 2013). LON can also act as a chaperone, independent of its proteolytic activity and promote the assembly of cytochrome c oxidase subunits. Thus down regulation of LON results in impaired respiratory function (Bota et al., 2005). Gibellini and colleagues (2014) showed that decreased LON levels impairs mitochondrial function (Gibellini et al., 2014b).

Further, this group also showed activation of executioner caspase 3 and apoptotic cell death as a result of LON down-regulation (Gibellini et al., 2014b). Our results are in agreement as we also show increased activity of caspase 3 (Table 2), a marker of apoptosis, involved in the execution of this process. Apoptosis may be a result of proteolytic defects of LON resulting in accumulation of oxidized proteins while loss of chaperone function impairs mitochondrial respiration.

Given that mitochondrial stress was induced by FA it seemed logical to assume that the enhanced activity of caspase 3/7 would be attributed to the intrinsic apoptotic pathway. However, the data presented in table 2 suggest that caspase 9 is not a good candidate for the up-stream activation of caspase 3/7 since this initiator caspase is slightly decreased in FA treated cells when compared to control cells. In the cytoplasm, cytochrome c binds to the APAf-1 and forms the apoptosome to induce the activation of pro-caspase 9 and initiate an enzymatic

reaction cascade leading to the execution of apoptosis via caspase 3 activity (Dai et al., 2014). Seeing that cytochrome c is an iron metalloprotein and absolute requirement for caspase 9 activity, the loss of iron from cytochrome c by FA mediated chelation can prevent the proper formation of the apoptosome leading to decreased activity of caspase 9. Furthermore, results have emerged providing evidence that Apaf-1 transforms ATP into ADP and is an ATPase and that ATP is essential for Apaf-1 binding to cytochrome c (Hu et al., 1999 , Chiarugi, 2005). Thus FA may prevent activation of caspase 9 by depletion of intracellular ATP.

The IAP family of proteins selectively binds and inhibits caspases to prevent apoptosis. All IAPs contain 1–3 BIR domains, which are cysteine and histidine rich and can fold into functionally independent structures that bind zinc (Zuo et al., 2012). The XIAP is the most potent member of this family (Makhov et al., 2008). The BIR2 domain of XIAP can bind to the amino terminal end of the small subunit of caspase-7 causing its inhibition, while the linker region accounts entirely for the inhibition of caspase-3. The BIR3 domain directly binds to, and inhibits caspase-9 through an amino-terminal end created by caspase-9 self-cleavage. The BIR1 domain displays no inhibitory activity. Essentially, the E3 ligase activity of the really interesting new gene (RING) finger domain allows XIAP to trigger ubiquitination of caspases 3 and 7, a process that represents one of the potential anti-apoptotic mechanisms of XIAP (Zuo et al., 2012). Studies conducted on the effects of zinc chelators and the expression of XIAP reveal XIAP depletion by destabilization of this metalloprotein and subsequent activation of caspases upon zinc removal (Zuo et al., 2012 , Makhov et al., 2008). The loss of function of the RING finger domain may also lead to enhanced caspase activity since ubiquitination is no longer possible. Thus, FA being an efficient chelator of zinc can interfere with XIAP stability and ubiquitination function, allowing apoptosis to proceed uninhibited by this IAP family member enhancing cell death.

Furthermore LON deficient cells switch to necrosis as a result of ATP depletion caused by mitochondrial defects. LDH release was increased - an indication of increased necrotic cell death (Figure 3.11). Thus, while apoptosis may be initiated after FA treatment the switch to necrotic cell death may be as a result of depleted ATP.

However, given that this leakage is a consequence of loss of cell membrane integrity, most likely caused by elevated oxidative stress, the role of the mitochondria in lipid synthesis cannot be dispelled. Indeed, experiments conducted by Schwer et al. (2006) clearly described a relationship between SIRT3 expression and the activity of acetyl CoA synthetase (Schwer et al., 2006).

McGuirk et al (2013) showed that glutamine metabolism can be dysregulated by the decreased expression of PGC-1 α . Their experiments revealed PGC-1 α and ERR α regulate both the canonical citric acid cycle (forward) and the reductive carboxylation (reverse) fluxes by modulating glutamine flux through the Krebs's cycle; the latter can be used to support *de novo* lipogenesis reactions (McGuirk et al., 2013). Given that FA was shown to down-regulate SIRT3 and PGC-1 α expression suggests that FA not only affects mitochondrial output but can also contribute to the dysregulation of other mitochondrial processes such as fatty acid metabolism.

The importance of fungi parasitizing agricultural produce is of concern due to their ability to produce mycotoxins. These toxins are often associated with disease states including tumorigenesis. Mycotoxins found to be contaminating maize are a health risk since maize forms part of a staple diet for many South Africans. High risk groups include subsistence farmers and poor rural communities as these groups have low socio-economic status and food security is often compromised.

An understanding of toxic mechanisms provides a basis between exposure and adverse health outcomes. Although some mycotoxins have been extensively studied and the pathways that give rise to their pathologies have been described, little is known about the effects of FA, particularly in human models. The results presented in this study describe the toxic mechanism of FA in a liver derived human cell line, providing evidence for hepatotoxicity that may aid in understanding and predicting the risks of FA exposure on the health of people and animals. Thus, these results will be important in food safety and may help raise awareness of FA toxicity as well as encourage good agricultural practises, both pre and post harvest , to limit the presence of this commonly neglected mycotoxin in maize.

Previous studies have implicated mitochondrial dysfunction and excessive production of ROS as possible mechanisms of FA induced toxicity (Jiao et al., 2014, Telles-Pupulin et al., 1998). The data of this study points to FA mediated loss of SIRT3 leading to the aberrant regulation of the mitochondrial acetylome regulatory network and shows that mitochondrial function and biogenesis is seriously impaired by FA and oxidative stress is increased. This provides an underlying cause for the observations made by previous researchers.

CHAPTER 5: CONCLUSIONS

5.1. Limitations of study

In vitro studies entail subjecting cells to doses of a toxin. The objective of *in vitro* studies is to determine the potential influences of such toxins, and to isolate them from other types of influences. However, a major disadvantage is that cells are removed from their natural environment, thereby eliminating the interaction and protection mechanisms otherwise available from the donor organism. In order to improve this study the use of a primary hepatocyte cell model and an *in vivo* mouse model will go a long way in establishing a holistic response to FA.

This study only made use of a 24 hour exposure period. Many proteins and stress pathways respond differently to toxins under acute and chronic conditions, therefore it would be interesting to determine the effects of FA in the HepG2 cell line after 6 hour and 48 hour exposure times to simulate acute and chronic exposures respectively.

This study did not assess the morphological changes to mitochondria in response to FA.

5.2. Conclusion

Taken together data of this study show that mitochondrial function and biogenesis is seriously impaired by FA. The results discussed above suggest that loss of SIRT3 leads to the aberrant regulation of the mitochondrial acetylome regulatory network and results in cellular damage and death. Our results point to loss of SIRT3 in response to this mycotoxin as a possible mechanism for cytotoxicity in the HepG2 cell line.

Oxidative stress responses such as mitochondrial protein quality control systems and proper regulation of biosynthetic processing in the mitochondria are essential for maintaining metabolic homeostasis and adaptation in cancer cells. Examination of cellular responses to mitochondrial stress and dysfunction after FA treatment provides useful information to unravel the molecular basis of this mycotoxins mechanism of action

The poverty problem in South Africa is largely rural, with subsistence farming playing a huge role in supplementing impoverished communities (Machethe, 28-29 October 2004). Maize can be infected with a host of toxigenic fungi including species of *Fusarium* which produce Fusariotoxins. Given that maize is an important agricultural commodity, particularly in rural South Africa, this study has provided some answers to the mechanism of FA toxicity and its implications for health.

5.3. Future studies

While the current *in vitro* study provided some answers to the molecular mechanisms involved in the mitochondrial dysfunction in liver cells after treatment with FA it cannot provide a holistic response. Therefore, the use of a primary cell line along with an *in vivo* mouse model will enhance the findings of this study and add to the body of knowledge surrounding the toxic potential of this mycotoxin. It will also be worthwhile to look at different exposure time periods to determine the toxicity of FA under acute and chronic scenarios.

Given that the liver is a key detoxifying organ it will be worth investigating the biotransformation of FA and its effects on the cytochrome system. The processing of FA in the liver will provide the information necessary to determine if any toxic metabolites are produced.

This study also alluded to fatty acid/lipogenesis dysregulation by FA. Seeing as the liver is central to lipid homeostasis.. This is of importance since perturbations to SIRT3 result in fatty liver. Experiments involving an *in vivo* mouse model to determine if FA affects acetylation status of key proteins involved in hepatic fuel metabolism are warranted. Such experiments could include looking at the expression profiles of these proteins before and after treatment with FA as well as looking at the morphology of the liver to determine pathogenicity.

Finally, seeing as FA affected mitochondrial function through SIRT3 it would be interesting to observe the effects of FA on the microRNA environment and how changes here can further manipulate SIRT3 regulation. Indeed, the microRNA miR-34a and miR-199a have been shown to regulate SIRT1 expression; however no studies have evaluated their implications on SIRT3.

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Appendix 1: Raw data for MTT

Table 1: Raw data used to determine the IC₅₀ value using the cell viability (MTT) assay.

<u>FA concentration</u> <u>(µg/ml)</u>	<u>Log FA concentration</u>	<u>Average absorbance</u>	<u>Cell viability (%)</u>
0	0.00	0.9201	100.00
25	1.40	0.8216	89.30
50	1.70	0.8619	93.67
100	2.00	0.4108	44.65
250	2.40	0.1402	15.24
350	2.54	0.1104	12.00
500	2.70	0.0346	3.76

Appendix 2: Caspase activity graphs

2.1.

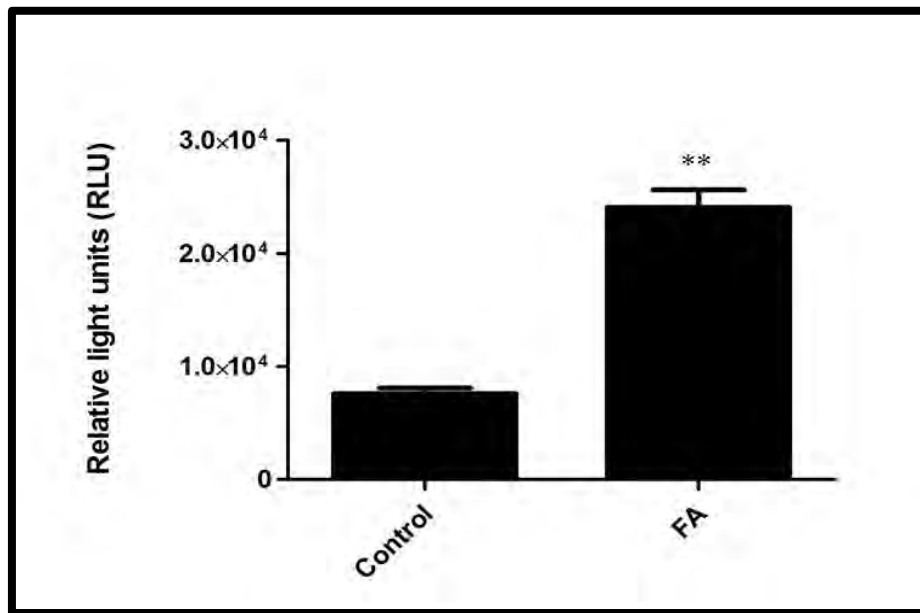


Figure 1: Caspase-3/-7 activity was significantly higher in cells stimulated with FA compared to untreated cells ($p=0.0032$).

2.2.

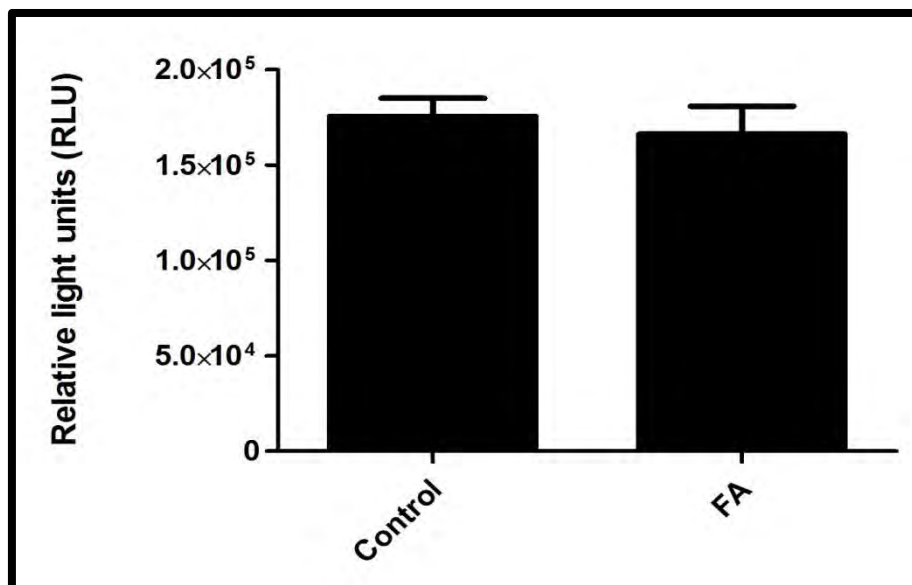


Figure 2: No significant decrease in caspase 9 activity was observed in treated cells.

Appendix 3: Standard curve for protein isolation

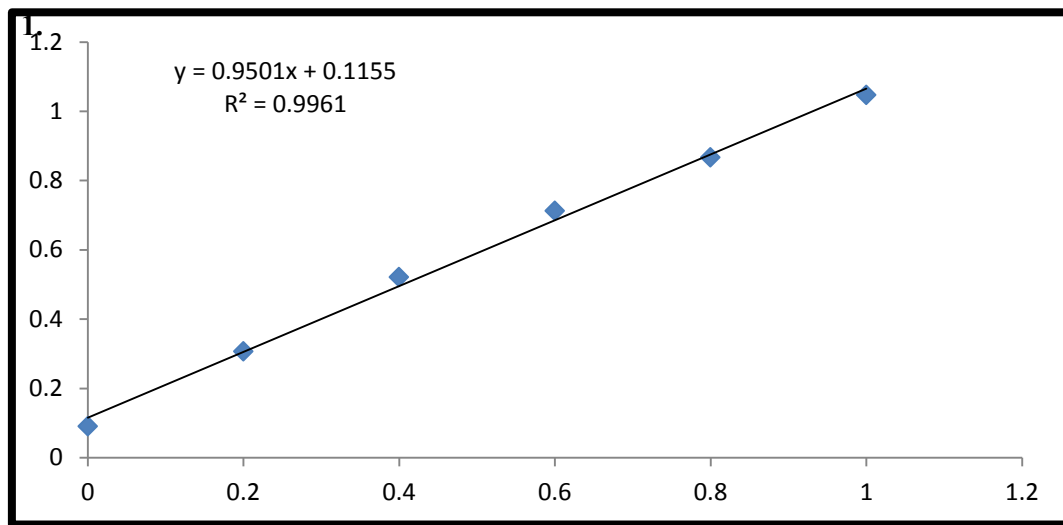


Figure 1: Standard curve using known concentrations of bovine serum albumin for the determination of protein concentration in samples using the bicinchoninic acid assay.

Appendix 4: SCGE results

1. Images for comet assay

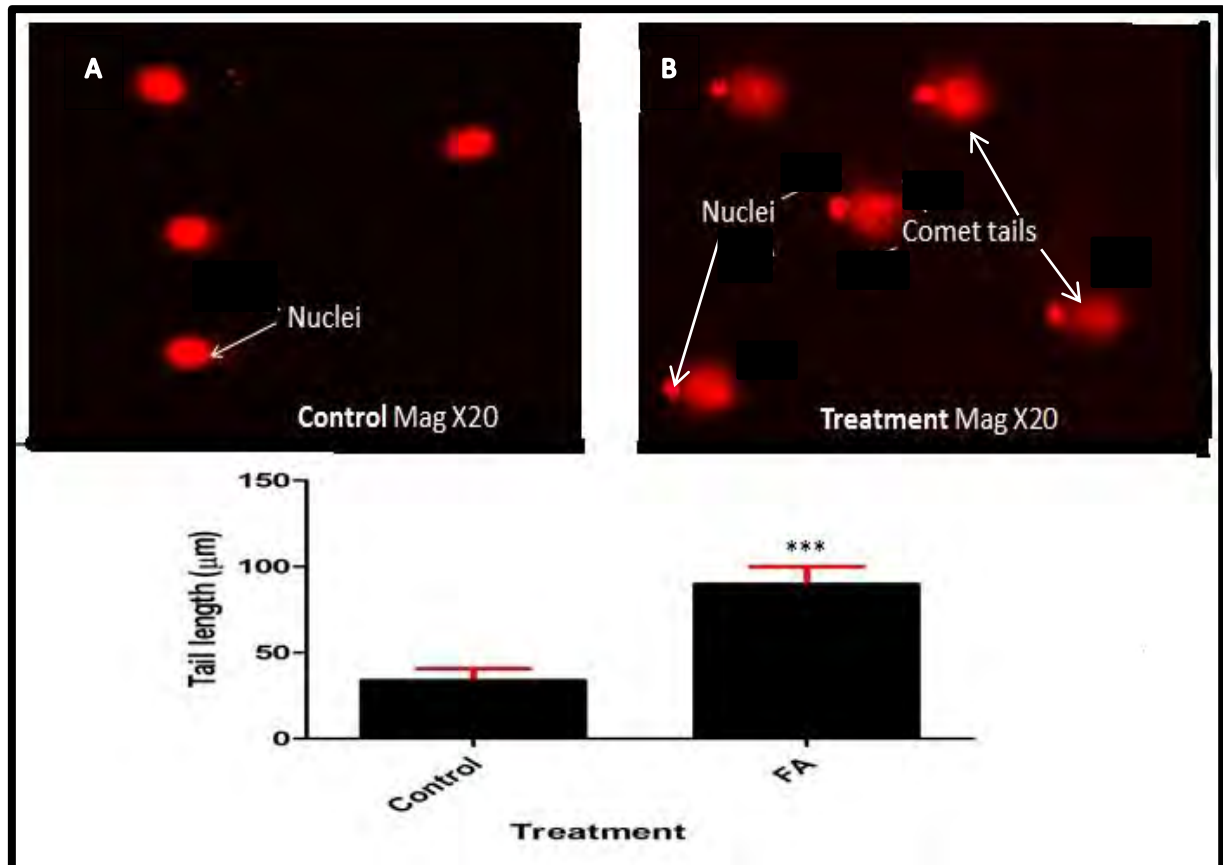


Figure 1: DNA fragmentation was markedly higher in cells exposed to FA (B) than untreated control cells (A) ($p < 0.0001$).